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PLANT PLASMALEMMA STRUCTURE:

AN IMMUNOLOGICAL APPROACH

PAUL MICHAEL NORMAN, B.A.

A thesis offered for the Degree of Doctor of Philosophy  
in Biology of the Open University.

July, 1986.

Date of Submission: 1<sup>st</sup> July 1986  
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DEDICATION.

In memory of my father,  
The Reverend John Norman  
1928-1983.

## ACKNOWLEDGEMENTS.

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Joachim Spiess and David Karr kindly gave advice on amino acid determination, and performed the analysis. Jim Jett and Mark Wilder of Los Alamos National Laboratory ran the flow cytometer and helped with data analysis, whilst Paul Jackson kindly provided laboratory facilities.

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# ABBREVIATIONS.

AGP	Arabinogalactan protein
AMP	Adenosine 5'-monophosphate
Ara	Arabinose
Asx	Aspartic acid or asparagine
ATPase	Adenosine 5'-triphosphatase (EC 3.6.1.3)
BSA	Bovine serum albumin (Fraction V)
CHAPS	3-[3-cholamidopropyl)-dimethylammonio]- 1-propanesulphonate
Con A	Concanavalin A
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GAG	Glucosaminoglycan
Gal	Galactose
GDP	Guanosine 5'-diphosphate
Glc	Glucose
GlcNAc	N-acetylglucosamine
Glx	Glutamic acid or glutamine
GS-I	Glucan synthase, type I (EC 2.4.1.34)
GS-II	Glucan synthase, type II (EC 2.4.1.34)
GTP	Guanosine 5'-triphosphate
HAT	Hypoxanthine, aminopterin, thymidine, deoxycytidine
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HT	Hypoxanthine, thymidine, deoxycytidine
Hyp	Hydroxyproline
IAA	Indole-3-acetic acid
K <sup>+</sup> -ATPase	Potassium-dependent adenosine 5'-triphosphatase (EC 3.6.1.3)
kd	Kilodalton
Man	Mannose
MES	2(N-morpholino)ethanesulphonic acid
Mg <sup>2+</sup> -ATPase	Magnesium-dependent adenosine 5'-triphosphatase (EC 3.6.1.3)
MS	Composition of Murashige and Skoog (1962)
NAA	Alpha-naphthaleneacetic acid
NP-40	Nonidet P-40
NPA	N-1-(naphthyl)phthalamic acid
PBS	Phosphate buffered saline
PBS-A-BSA	Phosphate buffered saline containing 0.1% sodium azide and 0.1% BSA
PMSF	Phenylmethylsulphonylfluoride
PTAC	Phosphotungstic acid-chromic acid
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine 5'-diphosphate
UML	Uchimiya and Murashige (1976) medium

#### PUBLISHED MATERIAL.

The material presented in Chapters 3, and 7 and the immunofluorescence microscopy experiments recorded in Chapter 4 have been published in *Planta* (Volume 167, pp 452-459, 1986). The results of Chapter 8 have been submitted for publication in *Planta*, whilst the experiments described in Chapters 5 and 6 form the basis of a paper in preparation.

# DISCLAIMER.

No part of this thesis has been submitted for any other degree of the Open University or any other University or Institution.

The thesis represents my own independent contribution, with the exception of the material presented in Chapter 8, which was performed in collaboration with Dr. M.S. Fitter (Plant Biology Laboratory, Salk Institute).

The flow cytometry experiments described in Chapter 4 used a machine maintained and operated by Dr. J.H. Jett (Los Alamos National Laboratory, Los Alamos, New Mexico). The amino acid analysis was performed by D.C. Karr (Peptide Biology Laboratory, Salk Institute). Cultures and plants of N. tabacum, N. plumbaginifolia and P. vulgaris were maintained by D.R. Lerner (Plant Biology Laboratory, Salk Institute).

# ABSTRACT.

Clonal hybridomas were generated which secreted monoclonal antibodies reactive with a crude membrane preparation from Nicotiana glutinosa suspension culture cells. Antibody secretion was assessed by a radioimmunoassay using such membranes as substrate. A number of the monoclonal antibodies recognised epitopes expressed on the external face of the plasmalemma of N. glutinosa suspension culture derived protoplasts, as assessed by immunofluorescence microscopy and flow cytometry. A second, non-overlapping set of antibodies recognised epitopes on the exterior face of the cell wall of intact cells, whilst a third group showed neither reactivity, and was postulated to recognise epitopes expressed within the cell.

Western blotting and immunoprecipitation analyses identified antibodies recognising a number of proteins including several plasmalemma glycoproteins. The recognised epitopes were periodate sensitive, and so probably in carbohydrate moieties. Immunoaffinity chromatography of a detergent extract of plant cells allowed purification of a plasmalemma glycoprotein. This was subjected to amino acid analysis, and used to raise polyclonal antisera and further monoclonal antibodies. Deglycosylation of a partially purified detergent extract of N. glutinosa cells suggested that this plasmalemma glycoprotein consists of a 50 kd molecular weight core protein, which is extensively and heterogeneously glycosylated, raising its apparent molecular weight to 130-230 kd.

The plasmalemma glycoprotein was used as an antigenic marker for plasmalemma derived vesicles resolved on sucrose density gradients, and for heterokaryons in protoplast fusions. Similarities between the plasmalemma glycoprotein and arabinogalactan proteins are discussed.

## **CHAPTER ONE.**

### **INTRODUCTION.**



The plasmalemma of higher plant cells has been postulated to be the site of a variety of biologically important functions, but there is remarkably little definitive information as to its properties. It will be argued here that this is due in major part to the force required to homogenise plant cells, to the close association of the plasmalemma with the cell wall, to the non-rigorous application of potential marker activities, and to the poor resolution of the separation techniques usually employed. The literature covering these aspects of fractionation, and the information which has been amassed will be extensively reviewed, and a number of alternative approaches which promise to improve the situation will be described. In particular, the potential of monoclonal antibodies as probes is assessed, in regard both to their use as specific markers for the plasmalemma of higher plant cells, and the successful application of this approach in elucidating the pathways of maturation of human T cells.

A number of activities have been assigned to the plant cell plasmalemma, often by analogy to animal, bacterial or fungal species, with no direct evidence to support the postulate. There is evidence, however, that the  $K^+$ -ATPase activity of plant cells represents the major proton extruding ATPase of plant cells, responsible for the transcellular electrochemical gradient which drives many active uptake mechanisms. The cellulose synthase machinery of the higher plant cell is also associated with the plasmalemma, as judged by electron microscopy. Other activities which have been postulated to occur at the plasmalemma include hormone action, phytochrome responses, disease resistance processes, stress responses, and events in establishment of symbiosis and sexual mating. Of these activities, only a few have been extensively studied, mainly those which have been postulated as plasmalemma marker activities. The published reports on these processes will be extensively reviewed, both with respect to their properties and proposed in vivo function, and its physiological significance. There is considerable dispute as to where these processes actually occur, and no unequivocal evidence of their plasmalemma association has yet been presented. Monoclonal antibodies

show considerable potential to resolve these ambiguities. This thesis concerns the production, characterisation and use of such antibodies as specific probes for the higher plant plasmalemma.

#### Plant cell structure and ultrastructure.

The studies of Hooke (1665) demonstrated the presence of cellular structure in cork and other plant tissues. With the advent of more refined staining, microscopy and electron microscopy techniques these studies have been extended to our present understanding of the structure of higher plant cells.

The prototypical plant cell is delineated by the plasmalemma, external to which is the polysaccharide-rich cell wall. A major proportion of the volume of the cell is taken up by the tonoplast-bounded vacuole. The remainder of the volume is taken up by the cytoplasm, which contains the nucleus and various other membrane-bound organelles, such as chloroplasts, mitochondria, Golgi apparatus and endoplasmic reticulum.

In sections of plant cells prepared for electron microscopy, the plasmalemma is seen to be intimately associated with the cell wall (Hall and Flowers, 1976), to have a pronounced lipid bilayer structure (Grove *et al.*, 1968), and to be the thickest of the cellular membranes-at least 8nm as determined by Morre and Bracker (1976). In freeze-fracture electron micrographs, many particles are seen, with sizes ranging from 7 to 15 nm (Willison and Cocking, 1972, 1975).

In 1969, Roland described a method for preferential staining of the plasmalemma in plant cell sections prepared for electron microscopy. Sections were treated with periodic acid, then stained with a solution of phosphotungstic acid in aqueous chromic acid. This was modified from a phosphotungstic acid-based staining procedure which preferentially labels animal cell plasmalemma, possibly by reaction with sialic acid residues of glycoproteins (Derner, 1973) or with phospholipids (Scott and Webb, 1975).

## Analytical fractionation of plant cells.

The phosphotungstic acid-chromic acid (PTAC) stain has been used as the basis for identification of plasmalemma-derived material in the analytical fractionation of membranes derived from numerous plant tissues. Most studies have followed a similar route in fractionation, in which an initial homogenate is subjected to differential fractionation to separate size classes of material, which are then subjected to step or continuous density gradient centrifugation to further purify components.

These techniques are again derived from those used on animal cells, but in their application to plant cells the cell wall presents an immediate impediment. In order to break open the cell wall and release the cell membranes, vigorous homogenisation methods are required. These result in loss of size differentials between organelles, so that it is difficult to specifically remove certain organelles by differential centrifugation techniques. Intact chloroplasts, mitochondria and nuclei as well as starch granules and large wall fragments should be precipitable by centrifugation at 10 000-13 000  $g_{av}$  for 10-20 min, leaving the bulk of microsomal membranes in the supernatant. These may be pelleted by centrifugation at 40 000-120 000  $g_{av}$  for 30-90 min, and will contain fragments from all cellular membranes, including broken components of those preferentially removed in the first centrifugation. Density gradient centrifugation may be rate-zonal, in which centrifugation is effected for a relatively short period, without membrane vesicles reaching equilibrium, when different membranes are separated mainly on the basis of the size of vesicles they form. Alternatively, isopycnic centrifugation may be used, in which centrifugation for a longer period allows vesicles to reach equilibrium when their density equals that of the medium.

The density at which this occurs is dependent on the nature of the density medium, of which sucrose is the most frequently used. This solute has a high osmotic potential, and a strong solvation effect,

which result in partial dehydration of particles in such a medium, and an increase in density. This can also have a detrimental effect on particle functions. In contrast, permeant solutes with lower solvation effects, such as glycerol, will cause membrane vesicles to equilibrate at lower density, similar to that of the solvated membrane in vivo. Renografin or metrizamide have also been used as gradient materials. These have low solvation effects and viscosities, the latter resulting in rapid attainment of density equilibrium. They do, however, interfere with a number of assays. Ficoll, in concentrations great enough for membrane vesicles to reach equilibrium, is very viscous, but has the advantage of low osmotic potential, and has been used as a gradient medium in combination with sucrose (Price, 1983).

All of these media, singly or in combination, have been used in studies of plant cell membranes. Because of their different properties, the medium used will affect the result of the experiment. Although sucrose has been most widely applied, the use of other media is highly significant, since experiments using them have not in general supported the consensus of experiments using sucrose gradients.

Typical studies on plant cell fractionation have involved homogenisation using razor blades, pestle and mortar, or mechanically driven shearing devices, in a buffered sucrose osmoticum at pH 7-8. The homogenate is usually filtered, then subjected to either low speed (ca. 1000  $g_{av}$ ) or intermediate speed (ca. 10 000  $g_{av}$ ) centrifugation, or both, followed usually by pelleting of microsomes at 40 000-120 000  $g_{av}$ . The microsomes are resuspended and resolved by isopycnic banding on a continuous or discontinuous sucrose density gradient for 2-20 h.

PTAC staining has been applied widely as a marker for plasmalemma-derived vesicles in fractionations as described above. Vesicles from individual density regions of a gradient are fixed, and stained as for sections of intact cells. The number of stained and unstained vesicles in a fraction is determined by examination of an electron micrograph, as an assessment of plasmalemma purity (Roland,

1978). Using this method, a number of activities have been shown by various authors to be coincident with maximum enrichment in PTAC stained vesicles.

Lembi et al. (1971) showed that for step gradient fractions, the binding capacity for N-1-naphthylphthalamic acid (NPA) was proportional to the percentage of PTAC stained vesicles in the fraction. NPA is a specific, potent inhibitor of auxin transport in plants, a process which is thought to be mediated by a plasmalemma localised transport system (Hertel and Flory, 1968).

Hodges et al. (1972), using step gradients, enriched 7- to 8-fold an ATPase from oat roots, which required  $Mg^{2+}$  for activity, and was stimulated by  $K^+$  or  $Rb^+$ . The peak of activity was coincident with the peak of PTAC stained membrane vesicles. This activity, and elaborations on it have been used extensively as markers for the plasmalemma in fractionation experiments.

Ray et al. (1969) demonstrated that incubation of density gradient fractions with low concentrations of UDP- $[C^{14}]$ glucose in the presence of  $Mg^{2+}$  resulted in production of beta-1,3- and beta-1,4-glucans. This activity (designated GS-I) was shown to coincide with the presence of morphologically identifiable Golgi-derived membranes. Subsequently, Van der Woude et al. (1974) demonstrated the presence of a second activity, of different buoyant density on sucrose gradients, which was active at higher UDP-glucose concentrations in the absence of  $Mg^{2+}$  ions. This activity, designated GS-II, correlated with the presence of PTAC staining vesicles in the gradient fractions.

Problems with plasmalemma markers.

One or more of the four assays described above have been used as markers for the plasmalemma in virtually all analytical fractionation experiments performed to date on plant cells, and a number of studies have sought to investigate the kinetics, ligand requirements and inhibition of the activities more fully. However, as was indicated

above, and has been frequently pointed out (Hall and Taylor, 1979; Quail, 1979; Hall, 1983), the use of NPA binding,  $K^+$ -ATPase and GS II as plasmalemma markers are all based on their correlation with PTAC staining. The suitability of these activities as markers thus rests on that of the PTAC stain.

#### Ambiguities in phosphotungstic acid staining.

Unfortunately, the specificity of the PTAC stain as a marker involves one assumption and a number of caveats. The assumption is made that the specificity shown by PTAC staining on sections of intact tissue is also shown on fractionated vesicles. This has never been proved; it would in fact be very difficult to do so. Further, there have been difficulties recorded in completely and uniquely staining the plasmalemma in tissue sections. Quail and Hughes (1977) found PTAC staining of prolamellar body membranes, lipid droplets and ribosomes in Cucurbita pepo hypocotyl hooks. Taylor and Hall (1978) found that the cell wall and plasmalemma of Nicotiana tabacum leaf cells stained inconsistently with silicotungstic acid (a PTAC-analogous procedure). Further, there was very poor staining of the plasmalemma of freshly isolated protoplasts, which improved if staining was performed 24 h after protoplast isolation. Robertson et al. (1978) found PTAC staining of the peribacteroid membrane in lupin root nodules, although they reported that staining of this organelle was variable.

Taylor and Hall (1979) used lanthanum chloride to stain the plasmalemma of N. tabacum leaf protoplasts, and demonstrated the lack of internal staining. After fractionation, lanthanum staining was also shown by vesicles from a sucrose gradient, but the distribution was not coincident with that of PTAC stained vesicles. PTAC also stained chloroplast granal lamellae, rough endoplasmic reticulum, smooth membranes and ribosomes. Hall and Flowers (1976) found that the plasmalemma was not stained with PTAC in areas where it was detached from the cell wall by limited plasmolysis.

Thom et al. (1975) found that PTAC stained not only the plasmalemma but also the tonoplast and endoplasmic reticulum in sugar cane suspension cells, advising great caution in applying the stain as a criterion for plasmalemma purity. They also noted that specifically stained sections of whole or parts of cells constitute a necessary control, but are rarely published in studies utilising the stain as a plasmalemma marker.

Nagahashi et al. (1978) pointed out that strict adherence to the protocol, and thorough washing were necessary for demonstration of staining specificity, and suggested the use of solutions at 38°C to increase staining intensity.

Additionally, the scoring of PTAC positive vesicles in fractions is prone to error. If, as is often the case, there is a gradation in staining intensity in vesicles, an arbitrary distinction must be made between stained and unstained components (Hall, 1983). It is also necessary to ensure that a representative proportion of the vesicles in a fraction is sampled (Quail, 1979). Wallach and Lin (1973) pointed out that a random distribution of particles to be counted cannot be reliably achieved. A sample of 1 mg dry weight might contain  $10^{10}$ - $10^{11}$  fragments of 1  $\mu$ m diameter. It would be difficult to score a significant proportion of these. It is also difficult to compensate for variations in size and mass of vesicles. A mitochondrion weighs ca.  $10^{-13}$  g whereas a smooth microsomal vesicle weighs ca.  $10^{-15}$  g. Thus a small number of the former in a population of the latter will present a significant impurity. Wagner et al. (1981) used measurement of vacuole size in photomicrographs to quantitate their volume. It might be necessary to apply such a method to membrane vesicles to accurately quantify their representation in a homogenate.

Finally, Quail (1979) has pointed out that the proportion of PTAC stained vesicles is equivalent to the specific activity in a fraction, and must be multiplied by the protein content to provide an estimate of absolute distribution between fractions. This calculation is rarely reported.

## Naphthylphthalamic acid binding to membrane fractions.

The binding of auxins and auxin inhibitors to plant cell fractions has been extensively studied. Hertel et al. (1972) showed binding of indoleacetic acid (IAA) and naphthaleneacetic acid (NAA) to Zea mays coleoptile homogenates. Batt et al. (1976) showed that there were two sites of auxin binding in this system. Batt and Venis (1976) resolved NAA binding on sucrose density gradients, and assigned the activities to Golgi or endoplasmic reticulum (site I) and to plasmalemma (site II). In contrast, Ray et al. (1977) resolved only one site in their homogenates. Ray (1977) localised this activity to the endoplasmic reticulum, whilst Dohrmann et al. (1978) assigned site II to the tonoplast rather than the plasmalemma on the basis of more thorough fractionation studies than those of Batt and Venis (1976). 2,4-dichlorophenoxyacetic acid binding showed different gradient distribution to either of the above sites, and was suggested to be to the plasmalemma. Ihl (1976) and Oostrom et al. (1975) found the major high affinity binding sites for NAA to be soluble and localised in the cytoplasm. The latter group pointed out that since the affinity of the soluble receptor was an order of magnitude greater than that of membrane-bound sites, the former was fully saturated in previous studies of the latter, and discounted as non-specific binding. Jacobs and Hertel (1978) found a third membrane-bound auxin binding site in Cucurbita pepo hypocotyls, which they assigned to the plasmalemma. From this discussion, it may be seen that the localisation and physiological significance of binding sites for auxins in tissue homogenates is far from clear.

Ray (1977) showed that the major binding site for NPA was on a membrane distinct from that binding NAA; he suggested that the former was the plasmalemma. Rubery and Sheldrake (1974) presented a chemiosmotic hypothesis of polar auxin transport, in which a pH gradient across the plasmalemma, with the exterior more acidic than the cytoplasm, allows cells to accumulate auxin. Cells are more permeable to the protonated form (HIAA) than to the anion (IAA<sup>-</sup>) ( $pK_a = 4.7$ ). The former would predominate in the extracellular space. At



the higher intracellular pH, the latter would be the predominant form, and would accumulate in the cytosol due to its lower membrane permeability. Plasmalemma-bound carriers for IAA<sup>-</sup> are hypothesised to be preferentially located at the basal end of the cell, resulting in preferential release of the hormone there, and polarity of auxin transport.

Jacobs and Gilbert (1983) raised a monoclonal antibody which inhibited NPA binding to pea stem cell membranes. In support of the above hypothesis, they showed by immunofluorescence microscopy that the antibody bound preferentially to the plasmalemma at the base of cells in stem sections. The results of immunofluorescence experiments on stem sections using the current antibodies (Appendix One) suggest, however, that specific binding of a monoclonal antibody to the plasmalemma is unlikely to occur under the conditions reported. The observed polarity would thus be an artifact of sectioning.

Apart from this paper, there are no independent determinations of the plasmalemma localisation of the NPA receptor. All other reports are based on the coincidence with PTAC staining, or with other activities, eg: GS-II, which are correlates of PTAC staining (Quail, 1979). Hall (1983) has pointed out that NPA binding may show quite a broad peak on density gradients (eg: Dohrmann *et al.*, 1978), and suggested that this may indicate that binding sites are not restricted to the plasmalemma.

#### Membrane bound ATPase activities.

The presence of numerous ATPase activities in plant cells has been extensively documented. Activity *in situ* and in tissue sections can be demonstrated by the method of heavy metal phosphate capture. Released phosphate from ATP hydrolysis is precipitated as the lead salt, by incubation in lead nitrate solution, and the localisation of the electron-dense lead phosphate examined by electron microscopy. Hall (1971) used this method to study the distribution of ATPase activity in Zea mays root tip cells. Activity was found associated with the

plasmalemma, nucleus, mitochondria, Golgi, vacuole and endoplasmic reticulum. The relative activities in each organelle varied for different cells of the section, but high activity was seen in the plasmalemma and plasmodesmata. Malone et al. (1977) performed an in vivo phosphate capture experiment, finding that the principal sites of deposition were the plasmalemma and mitochondria. Both mitochondrial and plasmalemma ATPase activities were inhibited by oligomycin. Edwards and Hall (1973) fractionated Zea mays root tip cells and examined the ATPase activity by non-denaturing gel electrophoresis, and heavy metal precipitation in the gel. Numerous activities were found, some of which were uniquely associated with specific organelles. Sullivan and Volcani (1974) found ten distinct ATPase activities in a marine diatom.

Whereas in animal cells the major plasmalemma ATPase is an electroneutral  $\text{Na}^+\text{-K}^+$  exchange pump, in fungal and algal cells it is an electrogenic proton pump. It seems likely that the same situation pertains in higher plants (Spanswick, 1981). Transport is stimulated by the presence of  $\text{K}^+$  in the extracellular medium, and it was postulated by Poole (1974) that the pump mediates  $\text{H}^+\text{-K}^+$  exchange. However, Bellando et al. (1979) showed that lipophilic cations would stimulate  $\text{H}^+$  excretion, but inhibit  $\text{K}^+$  uptake, indicating that electrical coupling of transport, rather than a common carrier might be the mechanism. Attempts to demonstrate proton pumping by microsomal fractions have shown little accumulation by plasmalemma-derived vesicles. This is due to leakiness of the vesicles to protons, as shown by the rapid decay of fluorescence quenching of 9-aminoacridine upon imposition of a pH gradient (Perlin and Spanswick, 1980). Mandala et al. (1982) found that the major uptake of methylamine in sucrose gradient fractions was by tonoplast-derived vesicles. DuPont et al. (1982) reached a similar conclusion for gramicidin-stimulated ATPase and ATP-dependent quinacrine quench in microsomal vesicles. The ability of the tonoplast to pump ions, coupled with the flow of current to adjacent cells via plasmodesmata (Spanswick, 1972) have made electrochemical studies of the plasmalemma of higher plant cells very difficult.

There has been some uncertainty as to what should be regarded as the plasmalemma-associated ATPase (Quail, 1979). The majority of reports use the  $K^+$ -stimulated  $Mg^{2+}$ -ATPase activity described by Hodges et al. (1972) as the plasmalemma marker. In contrast, others have reported the total  $(K^+ + Mg^{2+})$ -ATPase activity, without subtracting the basal  $Mg^{2+}$ -ATPase activity (eg, Leonard and Van der Woude, 1976; Nagahashi et al., 1978; Yoshida et al., 1983). There have also been reports where only the  $Mg^{2+}$ -ATPase was recorded (eg, Galbraith and Northcote, 1977; Kasamo and Yamaki, 1976).

The absence of a specific inhibitor for the plasmalemma ATPase (as ouabain is for the  $Na^+ - K^+$  pump of the animal cell plasmalemma) has been a handicap in dissecting the activity from the other ATPases present in the plant cell. Vanadate is a potent inhibitor of animal (Cantley et al., 1977) and fungal (Bowman and Slayman, 1979) plasmalemma ATPases, being active at less than 5  $\mu M$ . Cocucci et al. (1980) demonstrated an effect of vanadate on proton extrusion,  $K^+$  uptake, membrane potential, and ATPase activity in higher plant tissues, but the concentration required was 50  $\mu M$  or greater, at which phosphatases are also inhibited (Saxe and Rajagopal, 1981). The vanadate sensitive component of the potassium stimulated ATPase has thus been used as a plasmalemma marker (eg: Poole et al., 1984; Gallagher and Leonard, 1982), but its utility is obviously reduced by the lack of inhibitor specificity.

Bennett et al. (1984) showed that gramicidin would stimulate two ATPase activities in microsomes from Beta vulgaris. One was assigned to the plasmalemma and the other to the vacuole. The latter showed considerably greater gramicidin stimulation. ATPase activity has been shown to be stimulated by Triton X-100 (Cross et al., 1978; Gomez-Lepe and Hodges, 1978). It is concluded that a proportion of the enzyme is unavailable for ATP hydrolysis, being on right-side-out ATP-impermeable vesicles. The activity measured in the absence of detergent is on inside-out or leaky vesicles (Quail, 1979). ATPase assays are not normally performed in the presence of detergent.

Chanson et al. (1984) have shown that a  $K^+$ -stimulated  $Mg^{2+}$ -ATPase is present on Golgi-derived microsomes from Zea mays coleoptiles. The pH optimum is 7.5, as opposed to 6.5. This is a possible source of interference with the measurement of the putative plasmalemma  $K^+$ -ATPase.

The extent of  $K^+$  stimulation of the  $Mg^{2+}$ -ATPase varies considerably between species. Thus, whereas the originally reported oat root ATPase is stimulated 2- to 3-fold by  $K^+$  (Leonard and Hodges, 1973), in most species stimulation is much lower. Nagahashi et al. (1978) found that barley root ATPase was stimulated only 8% by  $K^+$ . The presence of plasmalemma-bound phosphatase activity rendered study of the  $K^+$ -ATPase impractical.

In conclusion, lack of verification of specific localisation of  $K^+$ -ATPase, independent of PTAC staining, and of its physiological role, coupled with the large number of potentially interfering ATPase and phosphatase activities, variations in the degree of  $K^+$  stimulation and the lack of specific inhibitors render the use of this activity as a universal specific plasmalemma marker dubious.

Nevertheless, there exists less direct evidence that the  $K^+$ -ATPase does represent a plasmalemma ion pump. This derives from studies of related enzymes in other species. Briskin and Poole (1983) found that incubation of beet cell homogenates at pH 6.5 with [ $\gamma$ - $P^{32}$ ]-ATP labelled a polypeptide of ca. 100 kd. There was rapid turnover of phosphorylation, which was vanadate but not azide sensitive; the rate of dephosphorylation was increased by KCl. Walderhaug et al. (1985) determined the amino acid sequence of phosphopeptides from the active sites of four ATPases.  $Na^+/K^+$ -ATPase from dog kidney,  $H^+/K^+$ -ATPase from pig gastric mucosa and  $K^+$ -ATPase from Zea mays roots had a common four amino acid sequence around the site of phosphorylation, which was similar to that of sarcoplasmic reticulum  $Ca^{2+}$ -ATPase. The sequence around the phosphorylated residue of Acholeplasma laidlawii plasmalemma ATPase differed. Serrano et al. (1986) found that the yeast plasmalemma ATPase had a similar sequence around the active

site, as well as eight other regions of homology with mammalian and E. coli ATPases. The activity was essential for viability in yeast. This implies that transport ATPases have highly conserved sequences, and that since the corn root  $K^+$ -ATPase shares a homology region, it is indeed a transport enzyme. Since this homology has only been demonstrated over four amino acids at the active site, however, stronger evidence that this enzyme is functionally homologous, and an intrinsic membrane protein, as has been shown for the other ATPases mentioned, requires more sequence information, such as could be gained by a complete cDNA sequence analysis.

#### Glucan synthase II activity.

The major problem with the use of glucan synthase II as a plasmalemma marker is the presence of an analogous enzyme on at least one other membrane system, namely the Golgi. Despite the difference in their kinetic properties, and requirement for  $Mg^{2+}$ , it is probable that conditions cannot be achieved which will allow one to be active but not the other, so that neither glucan synthase can be used as an absolute marker for its respective membrane (Ray, 1979). The pH optimum of GS-II may be quite narrow (Larsson et al., 1984), so that conditions must be carefully chosen to optimise activity.

Ray (1979) also reported the presence of a third glucan synthase activity which has similar properties to GS-II, but is present in both GS-I and GS-II containing fractions. Robinson and Glas (1983) showed measurable GS-II activity in Golgi-rich fractions from sugar cane cells.

The nature of the product also changes with UDP-glucose and  $Mg^{2+}$  concentration: higher UDP-glucose leads to more beta-1,3 linkages (Raymond et al., 1978), whilst higher  $Mg^{2+}$  concentrations favour more beta-1,4 linkages (Tsai and Hassid, 1971), and greater total incorporation (Henry and Stone, 1982a). These authors found that digitonin solubilised beta-1,4 and beta-1,3:1,4 glucan synthases from ryegrass suspension cultures, whilst digitonin-insoluble enzymes

produced beta-1,3 glucans in addition (Henry and Stone, 1982b).

That the conditions used to measure GS-II are either optimal or specific is far from proven. A variety of different assay methods and product washing regimens have been used, so that results of different workers are not necessarily comparable, as the same products are not necessarily formed or measured.

Ephritikhine et al. (1980) showed that GS-II was activated by increasing sucrose concentrations. Thus, if the activity is measured on sucrose gradient fractions, the peak buoyant density is overestimated. Correction for this effect shifts the peak value to lower densities. Robinson et al. (1982) showed an increase in peak density for GS-II activity when  $Mg^{2+}$  concentration was increased from 0.1 to 3mM. No change in density of Golgi membranes was seen.  $Mg^{2+}$  shifts are normally considered to be an exclusive property of vesicles from the endoplasmic reticulum (Lord, 1983).

The reason for the presence of glucan synthase activity on Golgi membranes remains obscure. It has been suggested that it may be involved in xyloglucan synthesis, or represent a cellulose synthase precursor (Green, 1983). Since the major products of GS-II are beta-1,3 glucans, it seems unlikely that this represents a cellulose synthase activity, which has only recently been isolated in active form (Callaghan and Benziman, 1984). This requires rather different conditions from those used to assay glucan synthases, including GTP,  $Ca^{2+}$ , exogenous cellobiose and a soluble factor for activity.

Anderson and Ray (1978) have shown the incorporation of glucose from UDP-glucose into membrane-bound beta-1,3 glucans in pea stem slices. They suggested that this was the result of activity of a surface localised callose synthase. The peak density was the same as for  $K^{+}$ -ATPase, which was taken as evidence of plasmalemma localisation. There was greater incorporation near the ends of segments, suggesting that the activity represented formation of wound callose by damaged cells. Pierce and Hendrix (1979), however, found

that this activity was not coincident with other plasmalemma markers. It has not been demonstrated that this represents in vivo activity of GS-II.

Haass et al. (1985) examined the orientation of GS-II in plasmalemma vesicles. Concanavalin A-sepharose chromatography was used to separate inside-out from right-side-out vesicles. They demonstrated that the synthase was present on the outside of right-side-out membrane vesicles, whilst inhibition by tunicamycin and amphomycin suggested the involvement of lipid intermediates, perhaps to transfer glucose from the cytoplasmic face. From detergent solubilisation experiments, however, Henry and Stone (1982b) concluded that lipid intermediates were unlikely to be involved, whilst Mueller and MacLachlan (1980) found evidence that the inner surface of cells must be exposed in cut sections for glucose from UDP-glucose to be incorporated into beta-glucans.

As for the other potential plasmalemma markers, the suitability of GS-II rests on that of PTAC staining. The presence of at least two activities, and uncertainties as to the optimum assay conditions further complicate matters. The in vivo significance of the activity remains obscure, whilst the overlap in assay conditions with GS-I and its sucrose stimulation render its distribution in gradients an unreliable plasmalemma marker.

Non-equivalence of plasmalemma markers.

Of the four assays for plasmalemma markers described above, three were identified by their coincidence with the fourth, namely PTAC staining. Of these three, two were demonstrated to be coincident only on step gradients. The use of such gradients rather than continuous gradients is a potential source of artifacts (Quail, 1979; Ray, 1979). The former degrades the continuum of the latter to a small number of discrete positions in which a given fraction may equilibrate. Thus, a step may trap more than one membrane and give the appearance of comigration of unrelated fractions. In addition, the steps have the

potential to occlude membranes at densities less than their true buoyant density (Green, 1983). The results of studies performed on step gradients are not necessarily confirmed when continuous gradients are used, and there are a number of reports of non-coincidence of combinations of the above markers.

Hendriks (1977) found that  $K^+$ -ATPase from maize coleoptiles was of at least two classes, showing heterogeneity in particle size and density. The same author (1978a) reported different distributions of GS-II and  $K^+$ -ATPase activity, concluding they were not uniquely associated with the same cellular membrane. A further report (Hendriks, 1978b) showed that the majority of the GS-II and  $K^+$ -ATPase activity from Zea mays coleoptile homogenates was found in the 10 000 g pellet. Density gradient centrifugation of the 10 000 g supernatant resulted in non-coincidence of these markers, whereas activities from the 10 000 g pellet comigrated on gradients, and were separable from cytochrome c oxidase. Nagahashi and Beevers (1978) found more than 40% of  $K^+$ -ATPase activity in a 250-8000 g pellet fraction.

Other reports have shown minor inconsistencies between the peak buoyant densities for the various markers. For example, Bowles and Kauss (1976) found rather little pH 6.0 ATPase activity in Phaseolus aureus hypocotyls, and this migrated as a broad peak at lower densities (1.12-1.15 kg/l) than those usually quoted for the plasmalemma (1.17-1.22 kg/l). They concluded that the localisation of the peak of this activity depended on the source tissue, being predominantly plasmalemma localised only in situations of rapid growth, such as seedling roots, but otherwise predominantly in the Golgi. In contrast, GS-II activity peaked at 1.17 kg/l.

Brett and Northcote (1975) found glucan synthase activity forming chemically similar products in all four fractions from a step gradient. It could not be assigned to a specific organelle.

Cross and Briggs (1976) reported an extensive study of the fractionation of marker activities.  $K^+$ -ATPase formed two peaks in



density gradients. One at 30% sucrose was not coincident with other plasmalemma markers, and was tentatively assigned to the Golgi or tonoplast. The second peak was at 38% sucrose and did not correlate with the peak of NPA binding. The latter was closely associated with cytochrome c oxidase, a mitochondrial enzyme, and it was suggested that NPA binding sites might be on this organelle.

When Leigh et al. (1975) fractionated Zea mays root homogenates on discontinuous sucrose gradients, they found a single peak of  $K^+$ -ATPase activity at 1.17-1.22 kg/l, in fractions which were enriched in PTAC staining vesicles. In contrast, on ficoll-sucrose gradients, a second peak of activity was seen at 1.08 kg/l, which did not show PTAC staining. Leonard and Van der Woude (1976) showed that upon centrifugation of ficoll-sucrose density gradients for 15 h rather than 1.5 h, the lower density peak diminished, and claimed that the higher viscosity of ficoll retarded the attainment of equilibrium by a fraction of plasmalemma vesicles. They presented evidence for the presence of PTAC staining vesicles in this fraction. It was not definitively shown, however, that recruitment into the higher density peak was occurring, rather than inactivation of the enzyme during the longer centrifugation period. There was substantial activity remaining at the lower density even after centrifugation for 15 h. These authors also showed that there was a linear relationship between percentage of PTAC staining vesicles and  $K^+$ -ATPase activity, although this did not extrapolate through zero, implying that these markers were not entirely confined to the same membranes.

Taiz et al. (1983) performed rate zonal centrifugation of Pisum sativum stem homogenates in renografin gradients. The peaks for  $K^+$ -ATPase, GS-II and NPA binding activity were clearly separated in the gradients. Boss and Ruesink (1979) also found non-coincidence of  $K^+$ -ATPase and GS-II peaks in isopycnic renografin gradients.

It thus seems that in addition to the reported non-equivalence of marker activities in sucrose density gradients, the use of other gradient media accentuates the effect, for reasons which have not been

fully explained. As has been pointed out (Quail, 1979), the equivalence of the marker enzyme activities would be expected, since all are correlates of PTAC staining. The numerous accounts of non-equivalence of these markers are more disturbing, and throw further doubt on the reliability of one or all of the plasmalemma markers. The uncertainty as to the suitability of these markers, coupled with the limited resolution of these activities from markers for other membranes (particularly mitochondria) on sucrose density gradients has severely limited the extent of available information on the plasmalemma, many studies being concerned solely with attempts to resolve the inconsistencies between these markers.

#### Other plasmalemma properties.

A number of further properties have been assigned to the plasmalemma and been used on occasion as markers. For example, the ratio of sterol to phospholipid is high in the plasmalemma of a number of animal cells, although probably not sufficiently characteristic to provide a useful marker (Wallach and Lin, 1973). This has, however, been used as a marker for plant cell plasmalemma. For example, Hodges *et al.* (1972) found a high sterol:phospholipid ratio in fractions enriched in  $K^+$ -ATPase activity. There have also been detailed reports on the lipid composition of potentially plasmalemma-enriched fractions (Travis and Berkovitz, 1980; Wright *et al.*, 1982), although the value of such figures for partially (ca. 80% by PTAC staining) purified fractions is dubious.

Others have detected cellulase in PTAC staining vesicle or  $K^+$ -ATPase rich fractions. Koehler *et al.* (1976) showed that this activity was stimulated 10- to 20-fold on treatment with Triton X-100. Pierce and Hendrix (1979) found a single peak for cellulase activity in sucrose density gradients, even under conditions where GS-II and  $K^+$ -ATPase showed two activity peaks. The *in vivo* role of the activity remains obscure; Bal *et al.* (1976) showed by immuno-electron microscopy that the majority of insoluble cellulase occurred on the inner face of the cell wall, in close association with cellulose

microfibrils, whilst no immunoreactivity was shown with the plasmalemma or Golgi.

Dieter and Marme (1980) demonstrated ATP dependent  $\text{Ca}^{2+}$  uptake into a crude microsomal fraction from Cucurbita pepo, claiming this to be plasmalemma-associated. This has not been proved, however, whilst Buckhout (1982) found that the rough endoplasmic reticulum was a major site of this activity.

The binding of the toxins fusicoccin and helminthosporoside has been assigned to the plasmalemma. Dohrmann et al. (1977) found that NPA and fusicoccin binding coincided in isopycnic gradients, but cytochrome c oxidase showed similar peak density, and so a unique localisation is not established. Strobel and Hess (1974) partially purified a helminthosporoside-binding membrane protein and raised antiserum to it. The evidence that the protein is plasmalemma localised is, however, equivocal.

It will be seen that the lack of definitive plasmalemma markers for higher plant cells, coupled to the poor resolution of isolation techniques has retarded the collection of additional information about structure and composition of this membrane. Although there is broad concurrence as to the equilibrium density between different approaches, their interreliance on PTAC staining degrades the quality of these correlations.

Recently, different separation methods for plant cell membranes have been used in attempts to improve the resolution of purifications, and more accurately define the properties of individual membranes. As well as their density and size differences employed in centrifugal separations, membranes also differ in their surface charge and hydrophobicity. The former property was used by Griffing and Quatrano (1984) as the basis for separation by isoelectric focussing in free solution of membranes from etiolated pea stems. Glucan synthase I and cytochrome c oxidase were each resolved as single peaks of unique pI. In contrast, GS-II formed two peaks of different pI, one more acid,

the other more basic than the GS-I peak. This result confirms others (Ray, 1979) in questioning the specificity of GS-II as a plasmalemma marker.

Two phase separations utilise both of the above mentioned properties, and have been investigated and reviewed by Larsson (1983, 1985). The quality of partitioning is dependent on the polymer concentration and salt content, which must be optimised for the species under study. Using a variety of species, Larsson (1985) and co-workers have obtained high plasmalemma purity, as assessed by PTAC staining,  $K^+$ -ATPase and GS-II, although the extent of enrichment of the different markers did not correlate. They also detected a light reducible cytochrome b in these fractions, which was also detectable in internal membrane fractions (Widell and Larsson, 1983; Widell et al., 1983). The protein (Kjellbom and Larsson, 1984) and lipid composition (Lundborg et al., 1983) of the enriched fraction were determined. There were about 40 polypeptides visible in polyacrylamide gels, whilst the lipid analysis indicated a high content of glycolipids.

Yoshida et al. (1983) combined a phase partitioning step with sucrose density gradient centrifugation to produce a plasmalemma enriched fraction from orchard grass seedlings. The  $(K^+ + Mg^{2+})$ -ATPase activity was detected as essentially a single peak on density gradients, although turbidity measurements and pH optimum of the ATPase suggested that there were other membranes present.

These procedures offer the potential for considerably more efficient separation of the plasmalemma from internal membranes, either by their use alone or in combination with density gradient centrifugation. The use of both separation techniques, as described by Yoshida et al. (1983) allows the fractionation of plant cell membranes using a wide range of different physical properties, and should increase the resolution obtained.

## Surface labelling of protoplasts or cells.

In an attempt to circumvent the ambiguities inherent in the methods described above, a number of reagents have been used in the hope of specifically labelling the plasmalemma either in thin sections or in isolated protoplasts. Protoplasts have the unique advantage of a freely accessible plasmalemma, allowing the use of weakly permeant or non-permeating ligands or macromolecules as potential labels. A number of low molecular weight ligands which have been used to label the plasmalemma of animal cells have been applied to plant protoplasts, whilst lectins and antibodies have been used as macromolecular labels.

Pyridoxal phosphate has been used as a potential plasmalemma label. Schiff bases formed with amino groups are reduced with tritiated sodium borohydride. The method works efficiently on animal cells (Hunt and Brown, 1974), but does not preferentially label plant plasmalemma (Hall and Roberts, 1975). Galbraith and Northcote (1977) found that the secretion of soluble acid phosphatases by Glycine max protoplasts precluded the use of this method. Cross and Briggs (1977) were unable to demonstrate surface specificity of fluorescamine labelling of corn coleoptile tissue slices.

Galbraith and Northcote (1977) and Perlin and Spanswick (1980) used diazotized sulphanilic acid to label protoplasts from Glycine max suspension cultures and Zea mays leaves respectively. The reaction is mild, occurring at neutral pH in 30 min. The labelling of protoplasts allows use of gentle disruption methods. Galbraith and Northcote (1977) fractionated their protoplast homogenates on continuous sucrose density gradients. They found that radioactivity from  $S^{35}$ -diazotized sulphanilic acid formed a broad peak in the gradient, which was coincident with one of two peaks seen for  $Mg^{2+}$ -ATPase, and also one for acid phosphatase. Unfortunately, this was not resolved from the peak of latent IDPase activity, whilst the width of the peak for radioactivity suggests some heterogeneity of labelled membranes. The viability of the protoplasts was judged to be at least 90%, and unaffected by labelling. There was some debris in the preparation,

however, which contained protein and lignin and so would be labelled also. Perlin and Spanswick (1980) used differential centrifugation of protoplast homogenates to isolate a plasmalemma enriched fraction, based on the distribution of specific activity of  $I^{125}$ -labelled diazotized sulphanilic acid. They claimed that this reduced contamination by Golgi, whilst there was little mitochondrial contamination. Incorporated radioactivity was clearly separable from latent IDPase on sucrose density gradients, whilst cytochrome c oxidase overlapped slightly.

Perlin and Spanswick (1980) did not report the quality of the initial protoplast preparation. This presents a problem, since the quality of the labelling is critically dependent on that of the protoplasts. This is because the labels used are not surface specific, so that specificity is generated by their inability to permeate the protoplasts in the preparation. Since only 2-3% of the total cellular protein is likely to be exposed on the external face of the plasmalemma (Quail, 1979), a small number of leaky protoplasts could produce a large artifactual binding, perhaps explaining the breadth of the peak seen by Galbraith and Northcote (1977). Juliano and Behar-Bannliier (1975) found that a proportion of mammalian cells in a population show strong internal labelling with exogenously added reagents. Galbraith and Northcote (1977) reported that soluble proteins were 44% of the total, but contained only 3% of the label in their experiment. This does not preclude the binding of cytosolic proteins to membranes during fractionation. No autoradiographic evidence was provided in either study that the label was surface localised before fractionation.

Iodination has been used extensively in studies of the plasmalemma of animal systems, but its application to plant sections or protoplasts has been particularly problematic. The method was applied by Yu et al. (1976) in an attempt to establish the site of membrane binding of the  $P_{FR}$  form of phytochrome in Zea mays coleoptiles. An earlier study (Yu, 1975) based on comigration in gradients of membrane-bound phytochrome, ATPase and PTAC stained vesicles concluded

that  $P_{FR}$  was bound to the plasmalemma. Because of the ambiguity of the markers used, and the low enrichment of PTAC stained vesicles in the peak fractions, Yu et al. (1976) used iodination of coleoptiles as a second criterion to identify the plasmalemma. The results indicated that the buoyant densities of membrane bound phytochrome and iodinated material were not identical. This was taken as evidence that the phytochrome was not plasmalemma associated.

Hendriks (1976) also used iodination of maize coleoptile segments to label a particulate fraction, which he identified as plasmalemma. Quail and Browning (1977) repeated the experiment on Cucurbita hypocotyls and found a similar peak buoyant density. However, by autoradiography they were able to show that the labelling was not at the plasmalemma but of an amorphous film at the cut surfaces and cuticle of the section, which it was suggested represented phloem exudate. They postulated that the labelled material became associated with a membrane fraction after homogenisation.

Attempts to specifically iodinate the plasmalemma of protoplasts have been similarly equivocal. Galbraith and Northcote (1977) were unsuccessful in an attempt to apply this technique, due to the high levels of endogenous peroxidase in their protoplasts. Substantial peroxidase activity has also been demonstrated histochemically (Hall and Sexton, 1972). In contrast, Schibeci et al. (1982) reported plasmalemma specific iodination of protoplasts from ryegrass suspension cultures. However, they provided no direct evidence for the plasmalemma localisation of the marker, and noted some penetration of the label into protoplasts. I was unable to demonstrate iodination of Nicotiana glutinosa protoplasts, presumably due to endogenous peroxidase activity as already mentioned.

A specialised case in which the plasmalemma of protoplasts has been successfully labelled was reported by Hornberg and Weiler (1984). Radiolabelled abscisic acid was photochemically cross-linked to high affinity binding sites on the plasmalemma of stomatal guard cell protoplasts of Vicia faba. The binding sites were trypsin sensitive

under conditions which left the protoplasts morphologically intact. Labelled proteins migrated in polyacrylamide gels with apparent molecular weights of 20.2, 19.3 and 14.3 kd.

#### Surface labelling with macromolecules.

Macromolecules possess a number of advantages over low molecular weight labels of protoplast plasmalemma. Their large size minimises the potential for non-specific labelling by diffusion into protoplasts, which is inherent with low molecular weight ligands. In addition, appropriate species may show considerable inherent specificity for the plasmalemma, whilst binding may stabilise the plasmalemma through subsequent homogenisation procedures. These advantages render this a potentially very useful method of identifying the plasmalemma, but one which has not so far been widely exploited. A number of lectins provide suitable labels of mammalian cell plasmalemma (Chang et al., 1975). Scarborough (1975) applied concanavalin A (Con A) to stabilise the plasmalemma of wall-less mutants of Neurospora crassa, on disruption of which the plasmalemma was isolated as large sheets, which could be collected by low speed centrifugation. Subsequent removal of Con A converted these into vesicles, which could be further fractionated. Con A binding to soybean protoplasts, causing agglutination, was observed by Williamson et al. (1976).

Boss and Ruesink (1979) labelled protoplasts from carrot suspension culture with  $[C^{14}]$ acetyl-concanavalin A. The stabilisation of the plasmalemma reported by Scarborough (1975) was not seen, but after homogenisation, labelled Con A was confined to a single peak of 1.14 kg/l in renografin gradients. Sucrose could not be used as a gradient medium, since it displaces Con A from its binding sites. Coincidence of Con A binding and  $K^{+}$ -ATPase was shown, although the latter was not confined to one peak. GS-II activity was separable from  $K^{+}$ -ATPase activity.



There have been several reports of the use of polyclonal antisera to agglutinate protoplasts. Hartmann et al. (1973) generated rabbit antisera using as immunogen lyophilised protoplasts of Vicia hajastana, Glycine max and Bromus inermis. Agglutination resulted in tight apposition of protoplast plasmalemmae. This was as efficient with antisera raised to the other species as with cognate sera, and could be enhanced by adding sheep anti-rabbit antibody, suggesting that it was antibody specific. Agglutination activity was only seen at x256 or lower serum dilutions.

Raff et al. (1980) raised antisera to leaf and suspension culture homogenates of Prunus avium. Antibody binding to protoplasts from pistil-derived callus culture was assessed by protoplast agglutination, immunofluorescence microscopy and rosetting of protein A coated sheep red blood cells. Agglutination by normal rabbit IgG was detectable, but was greater with IgG from immune sera. Immunofluorescence was specific for immune IgG on protoplasts, and did not occur with normal IgG on protoplasts, or with either normal or immune IgG on cells. Rosetting was also stronger with immune than non-immune sera, and occurred on protoplasts but not cells. The antibodies produced against pistil callus agglutinated protoplasts more effectively than did antibodies raised against organised pistil tissue. This suggests that the callus displays a broader range of surface antigens than the parental tissue, in support of an earlier report (Raff et al., 1979). In this study, cross-reaction of antisera raised against tissue homogenates of Prunus avium stems, leaves, pistils and anthers, and callus derived from them was tested by immunodiffusion and immunoelectrophoresis. Some antigens were unique to specific organs, and callus derived from them, whilst others were common. Callus cells expressed a wider range of determinants than did parental tissues.

The use of agglutination as an assay for antibody binding is called into question by results reported by Larkin (1977). Rabbits were immunised with Nicotiana tabacum protoplasts, and agglutination titres compared for control and immune sera. It was found that the

controls had at least as high an agglutinating titre as did immune sera, both against N. tabacum and Triticum aestivum and Avena sativa protoplasts. This agglutination was seen with non-immune sera from a variety of species including cat, goat, swamp wallaby and hairy-nosed wombat. It was concluded that agglutination was induced non-specifically by a normal serum component. Yariv antigens-synthetic glycosides with three functional saccharide moieties (Yariv et al., 1962)-containing beta-D-glucose, but not alpha-D-glucose or beta-L-glucose were also effective agglutinins. A further report (Larkin, 1978) demonstrated Yariv antigen induced agglutination of protoplasts of a wide variety of other species. It was concluded (Larkin, 1977) that a major serum globulin was responsible for the non-specific agglutination, by interaction with beta-lectins expressed on the plasmalemma, casting doubt on the use of agglutination as an assay for anti-plasmalemma antibody production in the above studies, and in that of Strobel and Hess (1974).

The major disadvantage of polyclonal antisera as reagents to investigate the plant plasmalemma has been the lack of specificity for this organelle. It would be expected that by immunising with crude homogenates of plant cells, antibodies to antigens from a variety of cell components would be raised. In this respect, it is perhaps surprising that Raff et al. (1980) found no cross-reactivity to walls of intact cells. Any attempt to produce a plasmalemma specific polyclonal antiserum is militated against by the poor resolution of homogenisation techniques, and uncertain identification of the plasmalemma in homogenates. In this respect, monoclonal antibodies have unique advantages, which are exemplified by their use to study surface properties of mammalian lymphocytes.

Use of monoclonal antibodies as surface probes of mammalian lymphocytes.

The experiments culminating in the production of monoclonal antibody secreting hybridoma cell lines by Kohler and Milstein (1975) were concerned with the elucidation of control of immune system functions, and immunologists were quick to realise the potential of the technique to further dissect these processes.

In many respects, this is a far more complex system than the dissection of plant cell structure. There are two separate lineages of lymphocytes (Warner *et al.*, 1962), namely the B cells responsible for humoral immunity by antibody secretion, and the T cells responsible for cell-mediated immunity and modulation of the immune response (Roitt, 1969). Both of these lineages may be subdivided into separate subpopulations, such as IgM and IgG secreting B cells and T-helper and T-suppressor T cells. These populations may be identified on the basis of immune system dysfunctions, but are morphologically identical. Polyclonal lineage-specific antisera may be generated by repeated absorption: eg, with T lymphocytes to generate B lymphocyte-specific sera (Raff, 1971). Cantor and Boyse (1975) generated polyclonal antisera distinguishing different T cell subclasses in mice, and were able to show that these became committed to express helper or killer activity before antigen was encountered.

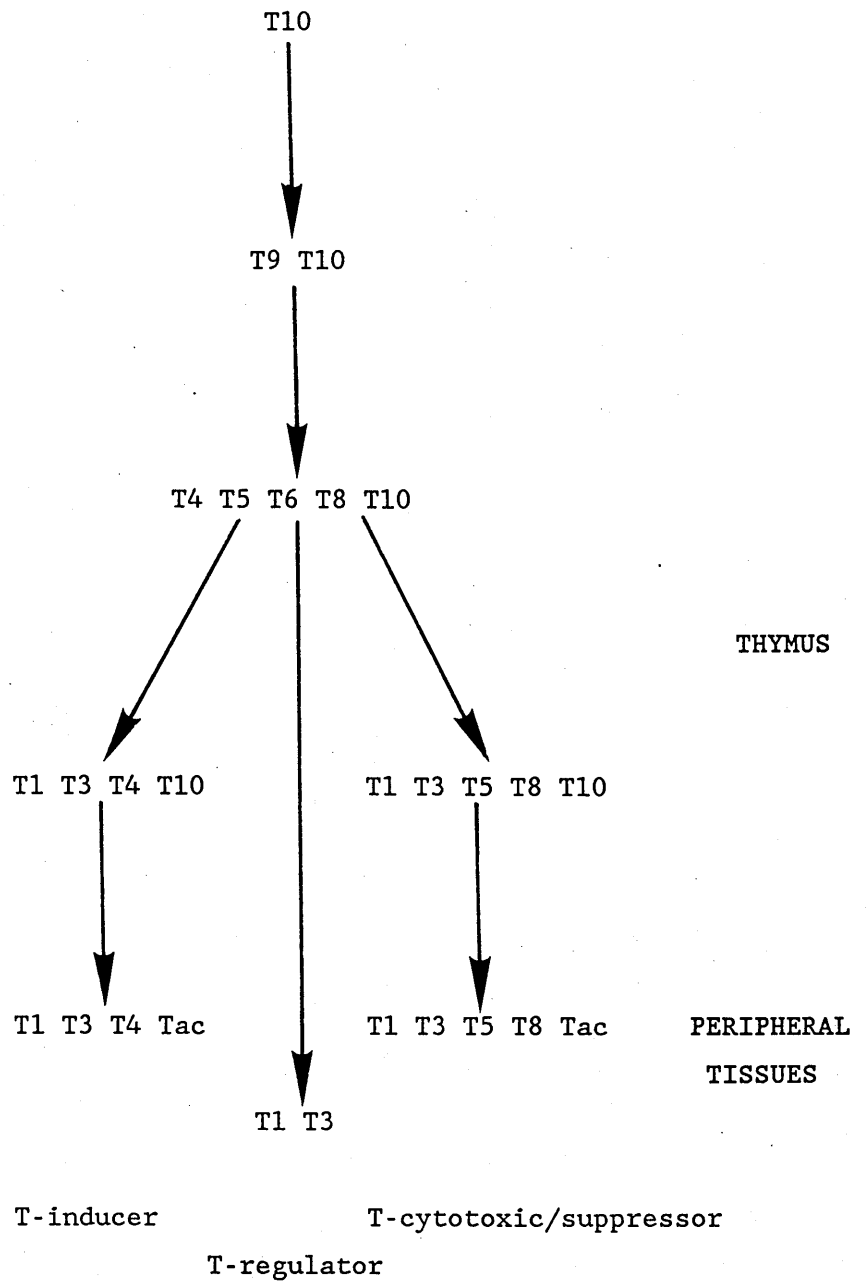
The use of monoclonal antibodies has allowed a substantial expansion and refinement of these studies. A number of groups have generated monoclonal antibodies which define the lineage and maturation of T cells in man (reviewed by Reinherz and Schlossman, 1980). This presents an impressive demonstration of the power of the monoclonal technique, since the manipulations used on mice, and the inbred lines on which to perform them are not available for humans. The maturation of T cells within the thymus can be followed with a panel of 8 monoclonal antibodies (Fig. 1.1). The earliest form of thymocyte expresses the monoclonal antibody defined surface antigen T10 only. These cells mature into cells expressing T9 and T10, then

express T4, T5, T6 and T8, but lose T9. This population of common thymocytes next differentiates into two subclasses, one of which expresses T1, T3, T4 and T10 ( $T4^+$ ), the other T1, T3, T5, T8 and T10 ( $T5^+$ ). These subclasses migrate from the thymus into the peripheral tissues, with loss of T10 antigen.

The different antibody defined populations of T cells may be positively selected by fluorescence activated cell sorting, and selected against by complement mediated lysis. Flow cytometry also allows quantification of populations with different fluorescent antibody binding features. This has allowed an assessment of the functional properties of the different populations.  $T4^+$  T cells possess an inducer-helper function in T-T, T-B and T-macrophage interactions, whilst  $T5^+$  T cells have cytotoxic and suppressor functions only. This dichotomy is programmed from the time of differentiation into antigenically distinct intrathymic populations. A further subset of T cells are  $T4^-T5^-$ , and are involved in feedback regulation of the other two classes.

Thus a panel of monoclonal antibodies raised against a heterogeneous population of intact T cells can define the development and differentiation of functionally distinct classes of morphologically identical T cells, based on their differential expression of plasmalemma antigens. None of these antibodies recognise activated and functionally mature human T cells exclusively. An antibody with this property, designated anti-Tac, was raised by Uchiyama et al. (1981). They further showed (Leonard et al., 1982) that this antibody recognised and functionally inhibited the plasmalemma bound interleukin-2 receptor of T cells. Further characterisation of the receptor (Leonard et al., 1983) culminated in the molecular cloning and sequence determination of this molecule (Leonard et al., 1984; Nikaido et al., 1984).

Figure 1.1. Monoclonal antibody defined maturation of human T cells.



This example illustrates the potential usefulness of monoclonal antibodies as probes for plant plasmalemma structure and function. First, it is possible to generate, using impure antigen preparations, specific probes for the external face of the plasmalemma. Second, these probes may define antigenically distinct subpopulations of morphologically identical cells, which may correlate with difference in their functions. Subpopulations may be isolated on the basis of their monoclonal antibody binding characteristics, and their properties studied. Third, the antigens recognised may be involved in the performance of cellular functions. It is possible for a monoclonal antibody to inhibit such a function, leading both to the elucidation of the significance of the function, and to the isolation of the molecule responsible for the function. This allows the molecular characterisation of the molecule in terms of primary structure and functionally important elements.

Such advantages could be very significant in the investigation of the plant cell plasmalemma. Monoclonal antibodies have the potential not only to specifically label the plasmalemma, but to define functions expressed there, and might define and isolate subsets of plant cells, such as those totipotent to divide and differentiate in culture, if this trait were correlated with expression of a specific plasmalemma epitope.

In the last few years, a small number of reports have been published describing the application of hybridoma technology to problems in plant cell biology. These will now be described to define the potential of the technique.

Use of monoclonal antibodies on plant systems.

Schibeci et al. (1982) reported the use of an iodinated monoclonal antibody which reacts with 1,6-beta-D-galactose residues to label the plasmalemma of protoplasts from ryegrass suspension culture. After labelling, the protoplasts were disrupted by osmotic/mechanical lysis, which resulted in formation of membrane sheets, similar to those

described by Scarborough (1975) from Neurospora, but which were not seen by Boss and Ruesink (1979) using Con A on soya protoplasts. These could be collected at low centrifugal force in step gradients, in a fraction enriched in I<sup>125</sup>-labelled antibody. There was no assessment of the purity of the fraction, however, save that the appearance and thickness of the membranes in this fraction were similar to those previously reported for plasmalemma in electron micrographs of intact cells. Protein bodies and starch granules were also visible.

Chin (1982) reported the production of monoclonal antibodies which inhibited or stimulated pH 6.5 ATPase activity in Hordeum vulgare cell suspension culture homogenates. No evidence was presented either that the effects on (K<sup>+</sup>+Mg<sup>2+</sup>)-ATPase were specific for one enzyme (see above) or that these antibodies reacted with the plasmalemma. A functional assay may not be an appropriate method to select monoclonal antibodies reacting with a single protein species. Weinshank and Luben (1985) found that monoclonal antibodies which inhibit cyclic AMP accumulation in mouse primary bone cells in response to bovine parathyroid hormone showed at least five classes of immunoprecipitation reactivity, selecting proteins of molecular weights between 18 and 180 kd.

A number of abstracts have been published outlining studies using monoclonal antibodies. For example, Galbraith and Maddox (1983) reported the production of monoclonal antibodies against regenerating Nicotiana tabacum protoplasts. Using an ELISA assay, they detected antigens the levels of which decreased, increased or remained constant between leaf and suspension culture protoplasts. ELISA was performed on fixed protoplasts, but the procedure took three days to complete, and used detergent and hypo-osmotic solutions, so that there was no evidence that surface localised determinants were detected.

In another abstract, Somerville and Sherwood (1985) reported raising monoclonal antibodies to Hordeum vulgare suspension cultures. 19 hybridoma lines were isolated which secreted antibody reactive with protoplasts. Immunofluorescence microscopy indicated that some of the

antigens recognised were expressed at the plasmalemma.

The best documented report is that of Brewin et al. (1985), who raised antibodies to the peribacteroid membrane from root nodules of Pisum sativum. One of these antibodies recognised the carbohydrate moiety of a series of glycoproteins, of molecular weight between 50 and 85 kd, as detected by Western blotting. Immuno-electron microscopy indicated that the antigen was expressed not only on the peribacteroid membrane, but also on the Golgi and plasmalemma. It was postulated that the determinant was generated in the Golgi, then transferred to the plasmalemma and peribacteroid membrane. It was also present in uninfected cells of pea, and in lupin root nodules and onion root meristem cell Golgi membranes. This epitope was immunodominant, being recognised by 6 out of 20 hybridomas generated.

Bolwell and Northcote (1984) generated monoclonal antibodies using endoplasmic reticulum and Golgi derived membranes partially purified on step gradients. Four of five hybridoma lines generated recognised the same series of 4 or 5 polypeptides on Western blots. A subclone from one of these hybridomas was found to inhibit arabinosyl transferase activity, which was tentatively assigned to a 70 kd polypeptide in the series.

#### Conclusion.

From the above discussion, it will be seen that there is considerable information as to the requirements and potential function of a number of putative plasmalemma associated enzyme activities. The weak link in such studies remains the lack of definitive evidence for their plasmalemma localisation. This has inevitably reduced their utility as plasmalemma markers, and their interest as plasmalemma associated enzymes, and limited the progress made in further elucidation of the properties of the plasmalemma of higher plants.

A number of promising alternative approaches to the purification and characterisation of the plasmalemma have been developed, and the



particular advantage of monoclonal antibodies as plasmalemma specific reagents has been noted above. In this study, the use of monoclonal antibodies is developed as a powerful tool to recognise the plasmalemma of higher plants. The production of a panel of hybridomas secreting monoclonal antibodies reactive to plant cell constituents is described, and evidence presented that a subset of these recognises epitopes expressed at the plasmalemma. These are used to localise the plasmalemma enriched fractions in isopycnic separations of plant cell homogenates. The nature of the recognised determinants, and their association with specific antigens is investigated, leading to the molecular characterisation of a plasmalemma associated plant cell antigen. In addition, the use of monoclonal antibodies as surface markers in cell fusion experiments is demonstrated.

Thus the work described indicates the potential for further characterisation of the plasmalemma of higher plants, for studies designed to investigate specific plasmalemma associated functions, and for the development of novel selection strategies for subpopulations of plant cells, and their genetic manipulation through somatic hybridisation.

## CHAPTER TWO.

### SOURCE AND MAINTENENCE OF PLANT MATERIAL.

## MATERIALS AND METHODS.

Source and maintenance of plant cell cultures.

### Nicotiana glutinosa.

The culture of Nicotiana glutinosa was a gift of Dr. David Radin, (University of California, Irvine) and was produced in the laboratory of Dr. Robert Leonard at the University of California, Riverside. The culture, designated as 7/81 was initiated in 1981 from a stem segment explanted from a plant grown under sterile conditions.

Cultures were grown on UMI medium (Table 2.1) as described (Uchimiya and Murashige, 1976), which was sterilised by autoclaving. The suspension culture was maintained by serial transfer every two weeks of ca. 5 g fresh weight of cells into 100 ml of fresh UMI medium in a 250 ml conical flask. Cultures were maintained in the dark at 27°C, under orbital agitation at 125 rpm. Stock callus cultures were maintained in parallel as a reserve in case of infection, by serial transfer at monthly intervals of three samples of ca. 1 g of tissue to ca. 30 ml of fresh medium containing 0.8% agar, in 10 cm petri dishes, and were grown in the dark at 27°C.

Table 2.1. Composition of UMI growth medium for *N. glutinosa*.

MS salts.

$\text{KNO}_3$	1900 mg/l
$\text{NH}_4\text{NO}_3$	1650
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
$\text{KH}_2\text{PO}_4$	170
$\text{Na}_2\text{EDTA}$	37.3
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	16.9
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6
$\text{H}_3\text{BO}_3$	6.2
KI	0.83
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025

30 g/l sucrose

2 g/l casein hydrolysate

100 mg/l myo-inositol

10 mg/l thiamine.HCl

10 mg/l pyridoxine.HCl

5 mg/l nicotinic acid

2 mg/l glycine

2 mg/l 2,4-dichlorophenoxyacetic acid

0.25 mg/l kinetin

pH 5.7

Nicotiana tabacum and Nicotiana plumbaginifolia.

Haploid callus cultures of N. plumbaginifolia were obtained from Unilever Plant Research (Colworth, Bucks.) and callus cultures of N. tabacum were initiated from sterilised seed. Both were maintained in the laboratory as callus and suspension cultures on MS medium (Murashige and Skoog, 1962; MS salts plus 30 g/l sucrose, 100 mg/l myo-inositol, pH 5.7) supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid and 0.5 mg/l 6-benzylaminopurine.

Petunia hybrida.

P. hybrida callus cultures were initiated from commercially available seed (cv Superior, Fredonia Seed Co., Fredonia, New York), which was surface sterilised for 10 s in 95% ethanol and 10 min in 5% sodium hypochlorite solution, washed thoroughly and soaked overnight in sterile distilled water. Imbibed seeds were placed on solid UMI medium on which they germinated and initiated callus after ca. 4 weeks. The most vigorously growing callus was selected for serial transfer and maintenance on UMI medium.

Phaseolus vulgaris.

Suspension cultures were available in the laboratory, where they were maintained on SHdpki medium as described (Dixon and Fuller, 1978).

Glycine max.

Soya callus cultures were maintained in the laboratory on B5 medium as described (Gamborg, 1982).

Triticum aestivum.

Wheat suspension cultures were grown on MS medium supplemented with 146 mg/l glutamine, 1 mg/l alpha-naphthaleneacetic acid and 1 mg/l 2-isopentenyladenine (Schaeffer et al., 1979).

Source and maintenance of whole plants.

N. glutinosa plants were grown from seed provided by the USDA Southern Regional Laboratory, Oxford, North Carolina. They were grown in soil at 27°C on a 14 h light:10 h dark cycle.

N. plumbaginifolia seeds were provided by Dr. Franz Hoffmann of the University of California, Irvine, and were grown as for N. glutinosa.

N. tabacum seeds were provided by Dr. Hoffmann, surface sterilised, and plants grown in vitro on MS basal medium (MS salts, 30 g/l sucrose, 100 mg/l myo-inositol), without hormones, at 27°C on a 14 h day:10 h night cycle. They were maintained by explant of sterile nodal sections every two months.

Phaseolus vulgaris plants were grown from commercially available seed (cv Improved Tendergreen, Burpee Seed Co., Warminster, Pennsylvania) as described for N. glutinosa.

Production of a total membrane preparation from plant tissues.

The method was based on that of Thom et al. (1975) and was applied to suspension cultures, callus and whole plant tissue. Suspension cultured cells were harvested by collection on miracloth filters (Calbiochem, La Jolla, California) and washed with 2-3 volumes of distilled water. They were extracted with a pestle and mortar at 4°C for ca. 5 min in 2-4 ml/g fresh weight of 250 mM sucrose, 25 mM Tris, 5 mM dithiothreitol, 3 mM EDTA, pH 7.4 (homogenisation buffer).

The extract was clarified by filtration through miracloth, and debris and unbroken cells removed by centrifugation at 1500  $g_{av}$  for 10 min at 4°C. The supernatant was then centrifuged for 1-1.5 h at 100 000  $g_{av}$  at 4°C. The pellet from this centrifugation comprised the total cellular membrane preparation, and was resuspended in a small volume of an appropriate buffer using a teflon-glass or glass-glass homogeniser. For immunological procedures, the buffer was phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l  $Na_2HPO_4$ , 0.2 g/l  $KH_2PO_4$ , pH 7.2), and aliquots were stored at -70°C until required; for further fractionation by density gradient centrifugation, homogenisation buffer was used. When necessary an aliquot was taken for determination of protein by the method of Lowry et al. (1951).

Protoplast production from N. glutinosa suspension cultures.

1) Sorbitol osmoticum.

Protoplasts were prepared from suspension cultured N. glutinosa cells 4-6 days after subculture, by a method modified after Nagata et al. (1981). Cells were harvested on a #60 mesh sieve and transferred to 1 ml/g fresh weight of protoplasting solution. This consisted of 1% cellulase Onozuka RS and 0.1% pectolyase Y23 (imported by Kanamatsu-Gosho Inc., Los Angeles, California) in an osmoticum composed of 0.4 M sorbitol, 3.3 mM MES, 0.1% BSA, pH 5.5. Protoplasting was for 2-3 h at 27°C with continual agitation on an orbital shaker at ca. 90 rpm. When protoplasting was complete as judged by the prevalence of rounded wall-free cells under microscopic examination, the protoplasts were harvested by filtration through a layer of 5-10 ml of loosely packed absorbant cotton wool in a 50 ml syringe barrel. This removed some of the undigested cells and debris from the suspension. The protoplasts were washed free of enzyme solution into an osmoticum appropriate for the intended use. The yield was  $2-4 \times 10^6$  protoplasts from 1 g of N. glutinosa cells in 10 ml of protoplasting solution.

## 2) Saline osmoticum.

Protoplasts were also prepared as above, except that the osmoticum used was that described by Meyer (1975), consisting of 2.5% KCl, 1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1% MES, pH 5.5. In this solution protoplasting proceeded faster and was essentially complete after 90 min. Protoplasts were harvested as described above; the yield was equivalent.

## Production of protoplasts from leaf tissue.

The method for production of protoplasts from leaf tissue was modified from that described by Haberlach *et al.* (1985). The composition of solutions is summarised in Table 2.2. Cellulysin and macerase were from Calbiochem (La Jolla, California). Leaves were cut from sterile *N. tabacum* plants and placed at 4°C in 150 ml of sterile conditioning medium for 24 h. Conditioned leaves were cut into 0.5-1 cm squares with a razor blade and placed in 40 ml of digestion medium in a sterile vacuum flask. A vacuum of 400-500 mm of mercury was applied for 2 min to infiltrate the enzyme solution into the leaf spaces and the flask then incubated at 28°C on an oscillating water bath at 40 rpm under fluorescent light for 14-16 h. The flask was swirled vigorously to release protoplasts into suspension, which was passed through #40 and #200 sieves to remove debris, and placed in Babcock bottles. These were centrifuged at 300  $g_{av}$  for 10 min. Because of the density of the sucrose solution, intact protoplasts floated in the bottles and collected in the neck, from which they were carefully removed with a pasteur pipette prior to washing in rinse solution by centrifugation as above. Washed protoplasts were withdrawn in a small volume of rinse solution and used in subsequent procedures. The yield was ca.  $5 \times 10^6$  protoplasts per 1 g of leaf tissue.

Leaf protoplasts from greenhouse grown *P. vulgaris* plants were produced similarly, except that they were immersed for 20 s in 75% (v/v) ethanol and washed 3 times in sterile distilled water prior to placing in conditioning medium.



Table 2.2. Composition of solutions for preparation of protoplasts from leaf tissue.

	Conditioning medium	Digestion medium	Rinse medium
KNO <sub>3</sub>	190 mg/l	190 mg/l	3800 mg/l
CaCl <sub>2</sub> .2H <sub>2</sub> O	44	44	880
MgSO <sub>4</sub> .7H <sub>2</sub> O	37	37	741
KH <sub>2</sub> PO <sub>4</sub>	17	17	340
Na <sub>2</sub> EDTA	3.73	3.73	9.32
FeSO <sub>4</sub> .7H <sub>2</sub> O	2.79	2.79	6.97
MnSO <sub>4</sub> .4H <sub>2</sub> O	2.2	2.2	5.6
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.86	0.86	2.2
H <sub>3</sub> BO <sub>3</sub>	0.62	0.62	1.55
KI	0.083	0.083	0.21
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.025	0.025	0.06
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.0025	0.0025	0.006
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.0025	0.0025	0.006
Casein hydrolysate	100		
Myo-inositol	10		
Nicotinic acid	0.5		
Glycine	0.2		
Thiamine.HCl	0.05		
Pyridoxine.HCl	0.05		
Folic acid	0.05		
Biotin	0.005		
Naphthylacetic acid		2	2
6-benzylaminopurine		0.5	0.5
Sucrose		137 g/l	137 g/l
Polyvinylpyrrolidone-40		20 g/l	
Cellulysin		5 g/l	
Macerase		1 g/l	
pH	5.8	5.8	5.8

## RESULTS.

### Growth of Nicotiana glutinosa in culture.

At the end of the subculture period,  $5 \pm 0.25$  g of cells were transferred to 100 ml of fresh UM1 medium. Cells were grown in the dark at  $27^{\circ}\text{C}$  and 60-70% relative humidity, agitated at 125 rpm on an orbital shaker. On successive days after subculture, three flasks were taken and the cells harvested on miracloth. The mass of cells was determined, and the pH and conductivity of the medium measured. The values of these parameters are shown in Table 2.3, and plotted in Figure 2.1.

Table 2.3. Growth characteristics of Nicotiana glutinosa suspension cultures.

Days after subculture	Mass of cells/g	pH	Conductivity/ mmho @ 27°C
0	2.2	5.7	6.0
1	1.6	5.8	6.2
2	1.8	5.8	6.1
3	2.3	5.5	5.7
4	3.3	5.1	6.0
5	3.9	5.2	5.7
6	4.2	4.9	4.5
7	7.6	5.0	5.8
8	13.5	5.1	5.2
9	16.4	5.0	4.8
10	23.3	5.1	3.9
11	31.1	5.2	3.1
12	37.1	5.4	2.4
13	34.7	5.5	2.7
14	38.2	5.5	2.1

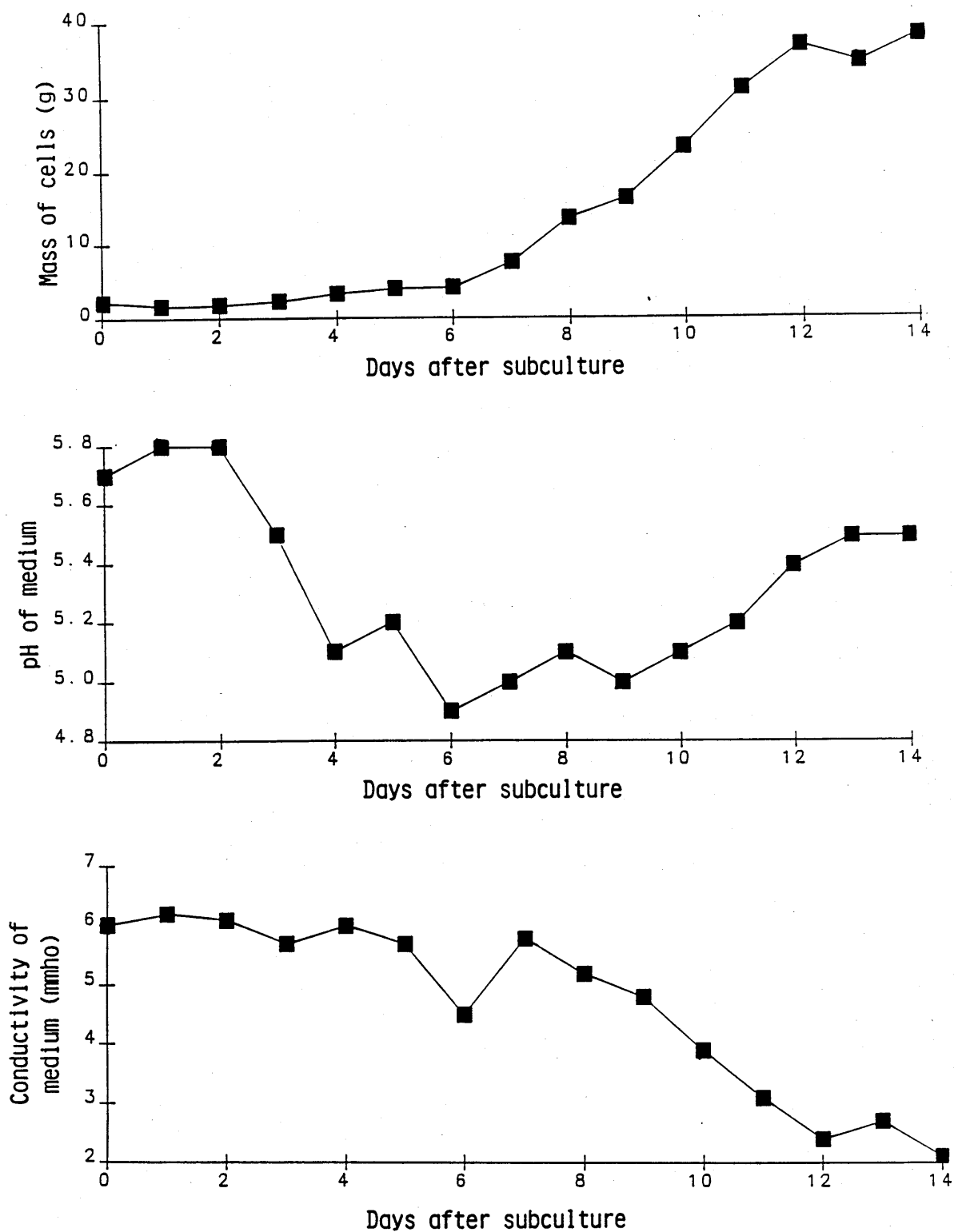


Figure 2.1. Growth characteristics of *N. glutinosa* suspension cultures.

Mass of cells (A), and pH (B) and conductivity (C) of medium were measured on consecutive days after subculture.

## DISCUSSION.

### Selection of plant material for study.

The suspension culture of Nicotiana glutinosa was chosen as the most suitable species and mode of growth for further study. The genus Nicotiana contains a number of species which have been extensively studied with regard to their genetics, properties in culture, protoplast production, somatic cell fusion and regeneration capacity, protein composition, and to a more limited extent their surface properties. Intact plants may be grown readily in a greenhouse, or explants grown under defined conditions in vitro.

Growth as a suspension culture affords rapid proliferation under highly controlled, axenic conditions on defined media. There is no light requirement for growth, so chloroplasts are not developed; chloroplast derived membranes may represent a major contaminant in microsomal membrane preparations from green tissues (Quail, 1979). The culture of N. glutinosa synthesised few phenolics, as evidenced by its white colour. Phenolic compounds can cause denaturation and loss of enzyme activity in cell fractionation experiments (Wetter, 1984; Loomis, 1974). A substantial mass of material for homogenisation may easily be obtained from a few flasks of cells-up to 40 g of cells per flask (Table 2.3). In addition, the suspension cultured cells of N. glutinosa are rapidly and efficiently converted to protoplasts using the enzymes cellulase Onozuka RS and pectolyase Y23. There is at least a 90% yield of protoplasts after 2-3 h of enzymic digestion. The rapid digestion limits the opportunity for changes in the metabolic activities and cellular protein composition due to osmotic shock and wall digestion (Fleck et al., 1982). The protoplasts are relatively stable and clean; there is little debris present and no residual cell wall as assessed by calcofluor white binding. These are important properties for the performance and correct interpretation of immunofluorescence microscopy studies on isolated protoplasts (Chapter 4), where repeated washing steps are necessary, and debris non-specifically adsorbed to the protoplast surface, or incomplete

cell wall removal could result in artifactual antibody binding.

A number of Nicotiana species show a high capacity to regenerate callus cultures from protoplasts from either leaves or suspension cultures. Callus cultures may be induced under appropriate hormonal stimulation to form shoots and roots, allowing regeneration of whole plants from a single protoplast (Takebe et al., 1971). This is potentially important both for somatic cell fusion and transformation experiments designed to assess the effect on the phenotype of the whole plant. Haploid plants and callus cultures of Nicotiana species may be generated by anther culture (Sunderland, 1984). Although these properties are not germane to the present study, they could be significant advantages in subsequent manipulations using monoclonal antibodies, and the antigens which they recognise. Unfortunately, the N. glutinosa culture showed low frequency of regeneration of cells from protoplasts, and no ability to regenerate shoots from callus, so such advantages could not be realised with this culture.

The properties of leaf protoplasts of Nicotiana glutinosa were not satisfactory for studies of antibody binding. Protoplasts were small, many lacked chloroplasts, and they were fragile. In contrast, leaf protoplasts from Nicotiana tabacum were more resilient, larger, and most contained numerous chloroplasts. They could be isolated in large quantities from leaf tissue of in vitro grown plants, with little associated debris. Thus they were the material of choice for studies using leaf protoplasts of Nicotiana species.

A major advantage of the use of suspension cultured cells as starting material was suggested by the studies of Raff et al. (1979; 1980). Callus cultures were found to express a wider range of antigens than did the organised tissues from which they were derived. Some of these antigens were common to callus from a variety of organs. Thus by using suspension cultures, it was judged likely that the hybridomas raised would secrete monoclonal antibodies which recognised epitopes with a broad tissue and species distribution. This would be a useful property in demonstrating unambiguously the location of the epitopes

recognised, and in interspecific cell fusion experiments. In addition, the probability of generating reagents of general utility in plasmalemma identification and fractionation is increased.

#### Selection of procedure for membrane isolation.

It was decided to use total cellular membrane preparations to immunise mice, for a number of reasons. First, as was discussed above (Chapter 1), there is no unequivocal marker for the plasmalemma of higher plants. Thus any procedure designed to enrich for this membrane might result in loss of a significant proportion of the desired material. By avoiding any reliance on available markers, it was hoped that the plasmalemma would be recognised without any a priori assumptions as to its nature or physical properties. Second, the use of monoclonal antibodies has the unique advantage of generating reagents specific for a single epitope confined to one or a few antigens, using a complex mixture of potentially antigenic molecules as starting material. This is a highly significant advantage over the use of polyclonal antisera or other reagents, and one which it was hoped fully to exploit in these studies. Third, any antibodies raised against determinants expressed intracellularly or on the cell wall provide useful controls to verify that any binding seen to the plasmalemma is antibody specific, and not an artifact of non-specific absorption of material from cell walls or broken protoplasts to the surface of intact protoplasts. Antibodies raised against internal membranes or the cell wall, and the antigens recognised might have intrinsic interest.

Because of the relative rapidity of the isolation procedure, the potential for degradation and denaturation of membrane bound molecules by any phenolics, proteases or lipases present in the cell is decreased. Thus the use of crude homogenates increases the possibility that the preparation used for immunisation of mice and characterisation of antibody specificity represents cellular membranes in a state similar to that in vivo.

These considerations led to the selection of Nicotiana glutinosa as a suitable species for study, suspension culture as an appropriate form in which to maintain the material, and from which to derive homogenates and protoplasts for the production and further characterisation of monoclonal antibodies, and a particulate fraction from a crude homogenate as the optimum form in which to present cellular membranes to mice to raise monoclonal antibodies. The subsequent chapter describes procedures used to generate hybridomas and to assay for antibody secretion. In addition, evidence is presented that the antigens recognised are of broad tissue and species distribution, as was anticipated in the above discussion.



## **CHAPTER THREE.**

### **PRODUCTION OF THE MONOCLONAL ANTIBODY LIBRARY.**

## INTRODUCTION.

The production of hybridomas secreting antibody against a given antigen is a complex process involving the interplay of a number of factors, including the nature of the immunogen, response of the mouse, and many variables in the fusion, selection and culture of the cell lines. This complexity has resulted in a plethora of variants on the basic technique, depending on the prejudices and experience of the laboratories involved. Some include elaborate manipulations, for which there is little proven basis, beyond the judgement of the investigator that they improve hybridoma yield. The technique used in this study was based on that used by Dr. Ian Trowbridge, which tends to omit superfluous manipulations, for example by not using feeder cells. Few variations on this protocol were attempted, so there is little ground for an assessment of the effectiveness of individual aspects of the procedure, the only criterion of effectiveness being the ability to raise hybridomas. Thus in this chapter the results of hybridoma production will be discussed with respect to the genetic, immunological and technical bases of the techniques, and also to the published results of other laboratories' manipulations, which are broadly similar to those described here.

## MATERIALS AND METHODS.

Production of hybridomas.

Growth media.

Mouse cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, composition as in Table 3.1) supplemented with 10% horse serum (HyClone, Logan, Utah) heat inactivated at 56°C for 30 min (DMEM-serum).

Selection for hybrid cells was in HAT medium, which is DMEM-serum supplemented with:

- 100 uM hypoxanthine
- 10 uM thymidine
- 2 uM deoxycytidine
- 1 uM aminopterin

Hybridomas were grown in HT medium, which is DMEM-serum supplemented with hypoxanthine, thymidine and deoxycytidine at the concentrations listed above, but lacking aminopterin.

Growth conditions.

Myeloma and hybridoma cell lines were grown in the dark in sterile petri dishes and multiwell plates placed in water-jacketed incubators (Napco, Portland, Oregon; Precision Scientific, Chicago, Illinois; VWR, San Francisco, California) at 37°C in an atmosphere of 13% CO<sub>2</sub> in air at ca. 100% relative humidity. All manipulations were performed in a sterile atmosphere in a laminar flow biological safety cabinet (NuAire, Plymouth, Minnesota), using short pipettes (Bellco Glass Inc, Vineland, New Jersey) and pasteur pipettes sterilised by autoclaving.

Table 3.1. Composition of Dulbecco's Modified Eagle's Medium.

Salts	NaCl	6400 mg/l
	NaHCO <sub>3</sub>	3700
	KCl	400
	CaCl <sub>2</sub>	200
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	200
	NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	125
	Fe(NO <sub>3</sub> ) <sub>3</sub> ·9H <sub>2</sub> O	0.1
Sugars	Glucose	4500
	Inositol	7
Amino acids	L-glutamine	584
	L-lysine.HCl	146.2
	Sodium pyruvate	110
	L-isoleucine	104.8
	L-leucine	104.8
	L-threonine	95.2
	L-valine	93.6
	L-arginine	84
	L-tyrosine	72
	L-phenylalanine	66
	L-cysteine	48
	L-histidine.HCl.H <sub>2</sub> O	42
	L-serine	42
	L-glycine	30
	L-methionine	30
	L-tryptophan	16
Vitamins	Choline chloride	4
	Folic acid	4
	Nicotinamide	4
	Pantothenic acid	4
	Pyridoxal.HCl	4
	Thiamine	4
	Riboflavin	0.4
Antibiotics, antimycotic, pH indicator	Phenol red	15
	Streptomycin	100
	N-butyl-	0.2
	p-hydroxybenzoate	
	Pencillin G	5x10 <sup>5</sup> units/l

Light microscopy.

Cultures were routinely observed in petri dishes and multiwell plates to monitor growth, using a variety of inverted microscopes, typically at x100 magnification using phase contrast objectives. Viable cells appeared smooth and brown, whilst dead cells were ragged-edged, smaller and appeared grey.

Determination of viability.

Determination of numbers of viable cells in cultures was by trypan blue exclusion. An aliquot of cells was diluted an appropriate amount into a solution of 0.16% (w/v) trypan blue in 0.85% (w/v) NaCl, and applied to a standard haemocytometer with counting chamber volume of 0.1 ul. Viable cells were detected by microscopy, based on their ability to exclude trypan blue.

Centrifugation.

Mammalian cells were separated from their growth medium in freezing and fusion procedures, etc. by centrifugation at 500  $g_{max}$  for 5 min at room temperature in a Beckman (Palo Alto, California) TJ6R centrifuge.

Source and maintenance of myeloma cell line.

S194/5.XX0.BU1 murine myeloma cell line (henceforth referred to as S194) was kindly provided by I.S. Trowbridge (Salk Institute). It is a bromodeoxyuridine-resistant mutant of a mouse myeloma which originally secreted IgA, but subsequently became antibody non-producing; it was isolated by R. Hyman (Salk Institute).

S194 cultures were maintained by transfer at weekly intervals of two drops from a pasteur pipette of an older culture into 6 ml of fresh DMEM-serum in a 6 cm petri dish. 5 days after transfer, the medium was aspirated to 2 ml, and 4 ml of fresh DMEM-serum added.

Upon receipt of the culture, it was grown up in 50 ml of DMEM-serum, and a number of ampoules cryopreserved as described below.

#### Immunisation of mice.

Fusions 16 and 17 were performed using 12 week old female Balb/c mice which were injected intraperitoneally three times at weekly intervals with total membrane extract equivalent to 2 mg of protein in 0.5 ml of PBS, followed a week later by a single intravenous injection equivalent to 1 mg protein in 0.25 ml.

Fusion 23 was performed using a 12 week old female mouse injected intraperitoneally twice with 4 mg of membrane protein, then once with 5 mg at weekly intervals. It was then rested for 4 weeks without further immunisation, after which a single intraperitoneal injection equivalent to 4 mg of protein was administered.

#### Fusion procedure.

Four days after the final injection, mice were killed by neck dislocation, surface sterilised for 2 min in 10% rocadyne solution (Rochester Midland, Rochester, New York), followed by 30 s in 95% ethanol, and their spleens removed with sterile scissors and forceps.

Each spleen was teased with sterile Graefe fixation forceps (Roboz, Washington, DC) for several minutes in 5 ml of DMEM, to release contained cells, and the suspension layered over 1 ml of horse serum in a 15 ml conical bottomed centrifuge tube (Corning, Corning, New York) to remove large pieces of connective tissue, which sank into the serum over 4 to 6 min. The DMEM layer containing the cell suspension was decanted and the cells pelleted by centrifugation and washed once in 5 ml of DMEM.

Prior to fusion, S194 myeloma cells were grown in the dark for 4 or 5 days to at least  $2 \times 10^7$  cells in 50 ml of DMEM-serum containing 100  $\mu$ M bromodeoxyuridine to select against revertants. Immediately

prior to fusion, they were harvested, and washed once with 5 ml of DMEM by centrifugation.

An aliquot of spleen cells was taken and freed of red blood cells by selective lysis in Tris-buffered ammonium chloride (140 mM  $\text{NH}_4\text{Cl}$ , 17 mM Tris, pH 7.2) prior to counting in trypan blue solution. S194 cells were also counted to determine total numbers and viability. Half the spleen cells (typically  $8-10 \times 10^7$ ) were combined with  $10^7$  myeloma cells in a sterile 40 ml conical bottomed glass centrifuge tube and centrifuged to pellet cells. Two tubes were prepared per mouse.

The supernatant was aspirated from the cells which were fused as below. All solutions were warmed to  $37^\circ\text{C}$  prior to fusion.

Time	Addition
0	1 ml 40% (v/v) polyethylene glycol in DMEM
1 min	0.5 ml 25% polyethylene glycol in DMEM
2 min	1 ml DMEM
4 min	2 ml DMEM
6 min	4 ml DMEM
8 min	8 ml DMEM

Polyethylene glycol (molecular weight 1500, British Drug House, Poole, Dorset; batch 5961400C) had previously been tested for fusigenic potential and the optimum concentration determined in trial fusions using unimmunised mice. The cell pellet was initially resuspended in the 40% polyethylene glycol solution by scraping with the pipette to break it into small clumps, and subsequent additions made gently down the side of the tube. Mixing was effected by gentle flicking of the tube with the finger, and was repeated occasionally between additions.

After the final addition, cells were collected by centrifugation, the pellet washed once in 5 ml DMEM then resuspended in 100 ml of HAT medium. 2 ml fractions were aliquotted into individual wells of two 24-well Linbro plates (Flow Labs, McLean, Virginia), which were placed

in the CO<sub>2</sub> incubator.

#### Growth of hybridomas.

At weekly intervals after fusion the wells were fed by aspiration of 1 ml of medium, and its replacement with 1 ml of fresh HAT medium. After 2 to 4 weeks, macroscopic colonies of actively dividing cells covered 10-50% of the bottom area of wells containing hybridomas. When the colonies had reached this density, an aliquot of supernatant (ca. 1 ml) was frozen for subsequent radioimmunoassay, and the cells picked into 6 ml of HT medium in 6 cm petri dishes. After 1-3 weeks, actively growing colonies covered much of the surface area of the bottom of their petri dishes, equivalent to  $2.5 \times 10^6$  cells, at which time they were frozen. Prior to this an aliquot was in some cases taken for cloning. An aliquot (ca. 5 ml) of the culture supernatant was retained for radioimmunoassay.

#### Cloning of hybrids.

Cloning by the method of limiting dilution was performed on cultures exhibiting a similar signal:noise in radioimmunoassay of the 6 cm petri supernatant to that of the 2 ml well from which it was picked. Cultures showing a large drop in signal were discarded.

Cloning was at 2 and 10 cells per well in the absence of feeder cells. An aliquot of an actively growing culture was counted in trypan blue solution to determine the concentration of viable cells. A 100-fold dilution was performed into 10 ml of HT medium, from which appropriate volumes were withdrawn to produce 250 and 1250 cells in 25 ml of HT medium: equivalent to 2 and 10 cells/well respectively when 200  $\mu$ l aliquots were dispensed to each well of a 96 well tissue culture plate.

Plates were incubated for 2-3 weeks, after which actively growing colonies were visible to the eye on the bottom of the well. When colonies covered 10-40% of the surface area of the bottom of their



wells, 100 ul of supernatant was taken for radioimmunoassay. Colonies showing positive signal:noise ratios were picked from their wells into 6 ml of medium in 6 cm petri dishes and grown for a further 1-3 weeks until they reached  $2-5 \times 10^6$  cells/dish, at which time they were cryopreserved. In some cases, colonies were immediately subjected to another round of cloning by limiting dilution.

#### Cryopreservation of cell lines.

Cultures of actively growing hybridoma cells were routinely cryopreserved in liquid nitrogen, as described by Galfre and Milstein (1981), in which state they may be stored indefinitely and thawed as required to regenerate actively growing cultures.

6 cm or larger petri dishes containing  $2-5 \times 10^6$  cells were harvested into 15 ml conical bottomed centrifuge tubes and the cells pelleted by centrifugation. The supernatant was aspirated, an aliquot being saved if necessary for radioimmunoassay. The pelleted cells were resuspended in 0.6 ml of cold ( $4^{\circ}\text{C}$ ) freezing medium, comprising 10% dimethylsulphoxide and 20% horse serum in DMEM. The suspension was transferred to a suitably marked cryotube (Nunc, Copenhagen, Denmark), and up to six cryotubes placed in individual one pint cardboard drums lined with cotton wool. These drums were placed at  $-70^{\circ}\text{C}$  for two or more days, after which the cryotubes were removed and transferred rapidly to liquid nitrogen.

To thaw cells, cryotubes were removed from liquid nitrogen and transported on dry ice to a  $37^{\circ}\text{C}$  waterbath, in which they were agitated for 1 or 2 min until thawed. The contents were placed in a 3 cm petri dish, and diluted at 20 s intervals with 0.6 ml aliquots of HT medium at  $37^{\circ}\text{C}$ , for a total of 2 min. The dishes were placed in the  $\text{CO}_2$  incubator for 2-18 h, after which most of the supernatant was withdrawn and fresh HT medium added. Cultures were transferred to 6 ml of HT medium in 6 cm petri dishes after active growth had ensued: usually 1-3 days.

## Iodination of antibodies.

Rabbit anti-mouse antibody (a gift from Dr Ian Trowbridge) was iodinated by the chloramine T method (Greenwood *et al.*, 1963). All procedures were performed in a fume hood equipped with a charcoal filter, behind a lead screen, whilst wearing a lead apron, isotope mask and double gloves. 10  $\mu$ l of antibody solution at 1 mg/ml was mixed in an Eppendorf tube with 20  $\mu$ l of 0.5 M sodium phosphate buffer, pH 7.2 and 2 mCi (4  $\mu$ l) of high specific activity carrier-free sodium iodide (Amersham, Arlington Heights, Illinois). To this was added 20  $\mu$ l of chloramine T solution at 1 mg/ml in water. The solutions were mixed and the reaction allowed to proceed for 5 min at room temperature, after which 20  $\mu$ l of 0.4 mg/ml tyrosine solution in water was added. After an additional 2 min, 50  $\mu$ l of foetal calf serum was added.

Iodinated antibody was separated from free  $I^{125}$  by gel filtration on an 8 ml Biogel P10 column (Bio Rad, Richmond, California) in a disposable 10 ml pipette, which had been pre-equilibrated with PBS-A-BSA, and was eluted with the same solution. 0.3 ml fractions were collected, the antibody typically being eluted as a sharp peak in ca. 1 ml after 3-5 ml of flow. The antibody peak was detected by gamma counting of 5  $\mu$ l of 1:40 dilutions of fractions showing detectable activity using a geiger counter. The peak fractions, typically containing  $0.5-2 \times 10^6$  cpm/ $\mu$ l, were pooled and stored in a lead container at  $-20^{\circ}\text{C}$  until required.

## Radioimmunoassay of antibody binding.

Supernatants from hybridoma cultures were routinely screened for the presence of antibodies directed against the total cellular membrane preparation by a liquid phase radioimmunoassay modified from that described by Morris and Williams (1975).

All steps were performed at  $4^{\circ}\text{C}$ . A vial of membrane preparation was thawed and diluted to 0.5 mg of protein/ml in PBS containing 0.1%

sodium azide and 1 mg/ml BSA (PBS-A-BSA). 50 ul aliquots were dispensed to each well of a 96 well V-bottom microtitre plate (Dynatech, Alexandria, Virginia), and incubated with 50 ul of hybridoma supernatant for 45 min. The plates were then washed three times by centrifugation at 1100  $g_{av}$  for 10 min, aspiration of the supernatant using a vacuum manifold adjusted to leave ca. 30 ul of residual liquid per well, resuspension on an orbital shaker at maximum speed for 2 min, and addition of 150 ul/well of PBS-A-BSA.

After washing, the membrane was resuspended and incubated with 50 ul/well of  $I^{125}$ -labelled rabbit anti-mouse antibody (prepared as below) diluted in PBS-A-BSA to  $1-5 \times 10^5$  cpm/well (100 or 200-fold). After incubation for 45 min, the plates were washed three times as above, the pellets resuspended in 100 ul PBS-A-BSA and transferred to transfer tubes (Skatron, Lier, Norway) for counting using a Beckman gamma counter, typically for 1 or 2 min/sample. Controls comprised PBS-A-BSA, one or more of a panel of monoclonal antibodies raised against Pseudomonas syringae var glycinae by V.P.M. Wingate (Salk Institute), and polyclonal immune and non-immune antisera diluted 1:1000 in PBS-A-BSA.

Results were calculated as a signal:noise ratio for the test clone relative to a hybridoma raised against Pseudomonas syringe var glycinae, typically the clone designated VC40.2B2, which usually bound 1000-1500 cpm/well of iodinated antibody.

#### Ascites production.

Ascites fluid was produced as a source of large quantities of antibody for selected clones, as described by Galfre and Milstein (1981). Ten 12 week old female Balb/c mice per clone were primed with a 0.4 ml intraperitoneal injection of 2,6,10,14-tetramethylpentadecane (pristane). One week later, they were injected intraperitoneally with 0.4 ml PBS containing  $5-10 \times 10^6$  hybridoma cells, which had been grown in 100 ml of HT medium, and washed free of medium in PBS.

7-10 days after injection of hybridoma cells, mice with productive tumours showed pronounced abdominal swelling. These were anaesthetised with metaphane and ascites fluid tapped from the peritoneal cavity by inserting an 18 gauge needle. If abdominal swelling reduced significantly after this procedure, the mice were left for several days before sacrifice, during which further ascites fluid was produced. Swelling which persisted after tapping, indicated that solid tumours were present, and mice were immediately sacrificed.

Prior to sacrifice, mice were anaesthetised, and their jugular veins opened to withdraw 0.5-1.5 ml of blood. Mice were killed by neck dislocation, the peritoneal cavity opened and ascites fluid withdrawn. Ascites fluid was clarified by centrifugation at 1000  $g_{av}$  for 10 min, and blood allowed to clot and centrifuged for 15 min at 15 000  $g_{av}$  after which the blood and ascites fluid were pooled and frozen at  $-20^{\circ}\text{C}$ .

#### Purification of antibody from ascites fluid.

This was performed as described in the EMBO-SKMB protocol (1980). Ascites fluid was diluted four-fold and mixed slowly with an equal volume of saturated ammonium sulphate solution at  $4^{\circ}\text{C}$ . After standing at  $4^{\circ}\text{C}$  for 30 min, the solution was centrifuged at 5000  $g_{av}$  for 10 min. The supernatant was removed and the pellets resuspended in 5 ml of 20 mM Tris, 40 mM NaCl, pH 7.8. The solution was dialysed overnight at  $4^{\circ}\text{C}$  against three changes of 2000 ml of 20 mM Tris, 20 mM NaCl, pH 7.8. The sample was centrifuged for 20 min at 15 000  $g_{av}$  at  $4^{\circ}\text{C}$  to remove denatured proteins, and the supernatant applied at room temperature to a 20 ml Whatman DE52 column. The column was washed briefly before elution with a linear gradient of 20-400 mM NaCl in 20 mM Tris, pH 7.8 over 3-4 h. The peak of antibody elution was detected by measuring  $\text{OD}_{280}$  of fractions diluted 200-fold, and confirmed by SDS-polyacrylamide gel electrophoresis of an aliquot of each fraction. Peak fractions were pooled, and the NaCl concentration adjusted to ca. 150 mM. The solution was filter sterilised and stored at  $4^{\circ}\text{C}$ .

Other hybridoma libraries.

Other libraries of hybridomas have been raised in the laboratory. M.S. Fitter and D.R. Lerner raised a series of clones against N. plumbaginifolia suspension cultures using immunisation, fusion, assay and growth conditions as described above. M.G. Hahn raised hybridomas against intact N. tabacum leaf protoplasts which were used for intraperitoneal immunisations, whilst a membrane extract from protoplasts was used for the intravenous immunisation. Clones were screened for secretion of antibodies directed against the outside of the plasmalemma by an enzyme linked immunoassay on whole protoplasts. In certain subsequent assays, data derived using these antibodies will be described. Clones from the N. plumbaginifolia library are designated Np, those from the N. tabacum library are designated Nt.

## RESULTS.

### Characteristics of the radioimmunoassay.

General features of the assay were ascertained using polyclonal antiserum collected by tapping the tail vein of mouse 16, and a control antiserum from an unimmunised mouse. Cells were allowed to form a clot and the blood clarified by centrifugation at  $15\,000\,g_{av}$  for 15 min. The clear supernatant was diluted 1:100 in PBS and used for the experiments.

Figure 3.1 shows a plot of extent of iodinated antibody binding versus concentration of membrane for a number of dilutions of polyclonal antiserum. From the results, membrane preparation equivalent to 0.5 mg/ml protein was chosen as an appropriate concentration at which a strong signal could be detected from immune serum at dilutions equivalent to the content of antibody expected in a hybridoma supernatant, whilst the requirement for membrane material was comparatively small. A subsequent substrate dilution curve (Fig. 3.2) using supernatants from a number of isolated hybridoma lines indicated that specific antibody binding could be detected at lower membrane concentrations for these clones. However, significant binding of supernatant from clones which secrete smaller amounts of antibody, or for which the recognised epitope is rare might be more easily detected with higher membrane concentrations.

Figure 3.3 shows a dilution curve for polyclonal antiserum at fixed membrane concentration. This indicates that a signal could be detected even at 3200 to 6400-fold dilution, and that its magnitude was logarithmically dependent on the antibody concentration. The extent of second antibody binding was influenced little by dilution of the non-immune serum, indicating that the background is probably mainly the result of non-specific binding of the second antibody to the membrane preparation.

A dilution of hybridoma supernatants (Fig. 3.4) yielded similar results for most clones, which behaved like the supernatant from hybridoma 16.4B4. Some supernatants could, however, be diluted 2- or 4-fold, with a slight gain in bound second antibody. The most extreme example is the supernatant from hybridoma 17.3A6, where dilution 1:4 in PBS resulted in a 50% rise in signal above the undiluted level. This suggests that the hybridoma secretes high levels of antibody which compete for available antigenic determinants. By lowering the antibody concentration, such intermolecular competition is reduced and the bound antibodies are able to fill both their active sites and so increase the avidity of their binding, and thus the amount of bound second antibody.

The characterisation experiments indicate that radioimmunoassay under the conditions used presents a valid method for detection of monoclonal antibodies, which is sensitive over quite a wide range of conditions. It was subsequently used to detect antibody secretion in the isolation of a number of hybridoma lines.

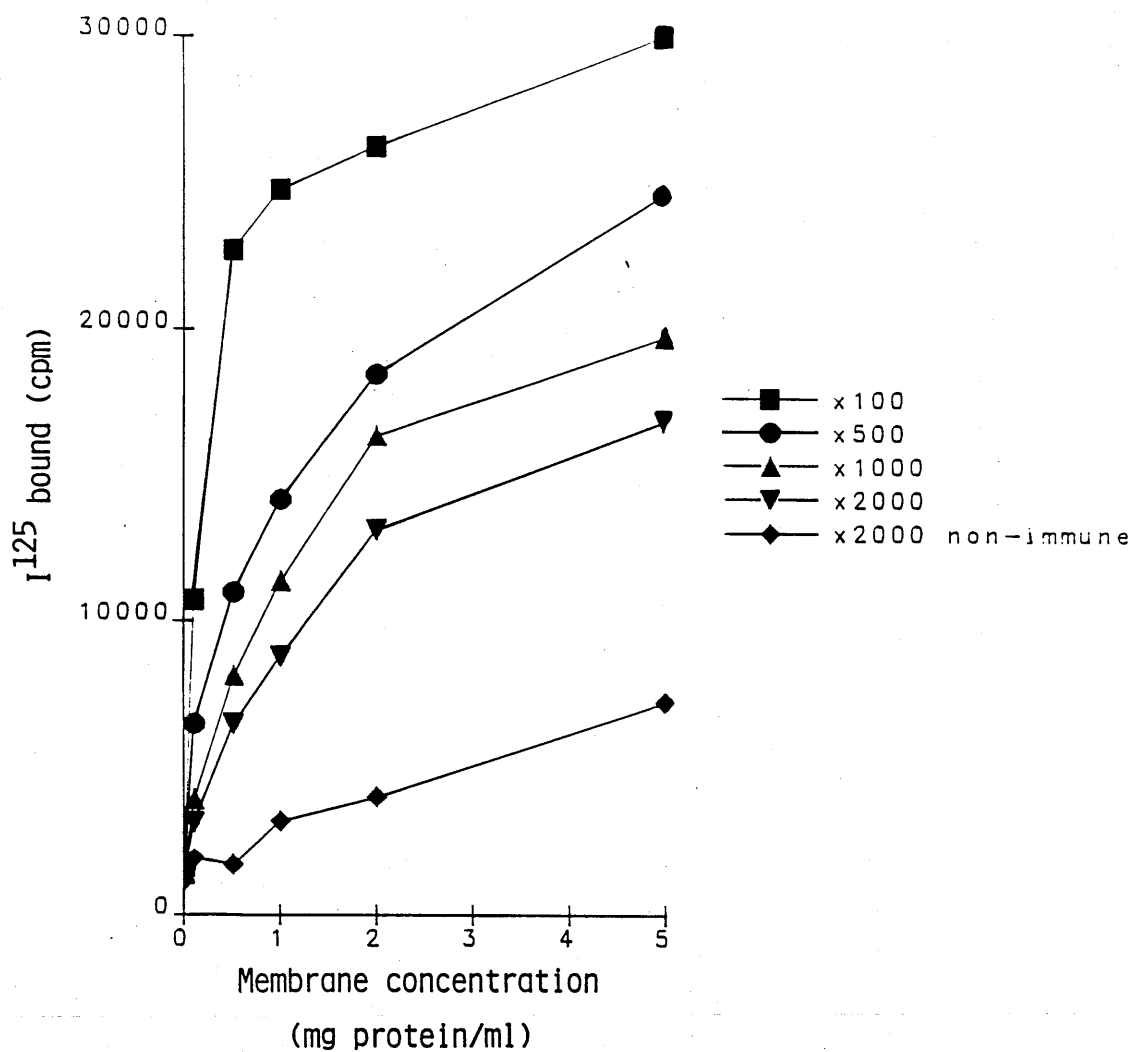


Figure 3.1. Membrane concentration dependence of radioimmunoassay signal for polyclonal antisera.

Polyclonal immune and non-immune sera were diluted as indicated with PBS-0.1% BSA. Antigen was diluted in the same buffer to 0.1, 0.5, 1.0, 2.0 and 5.0 mg protein/ml, and used as the substrate in the assay.



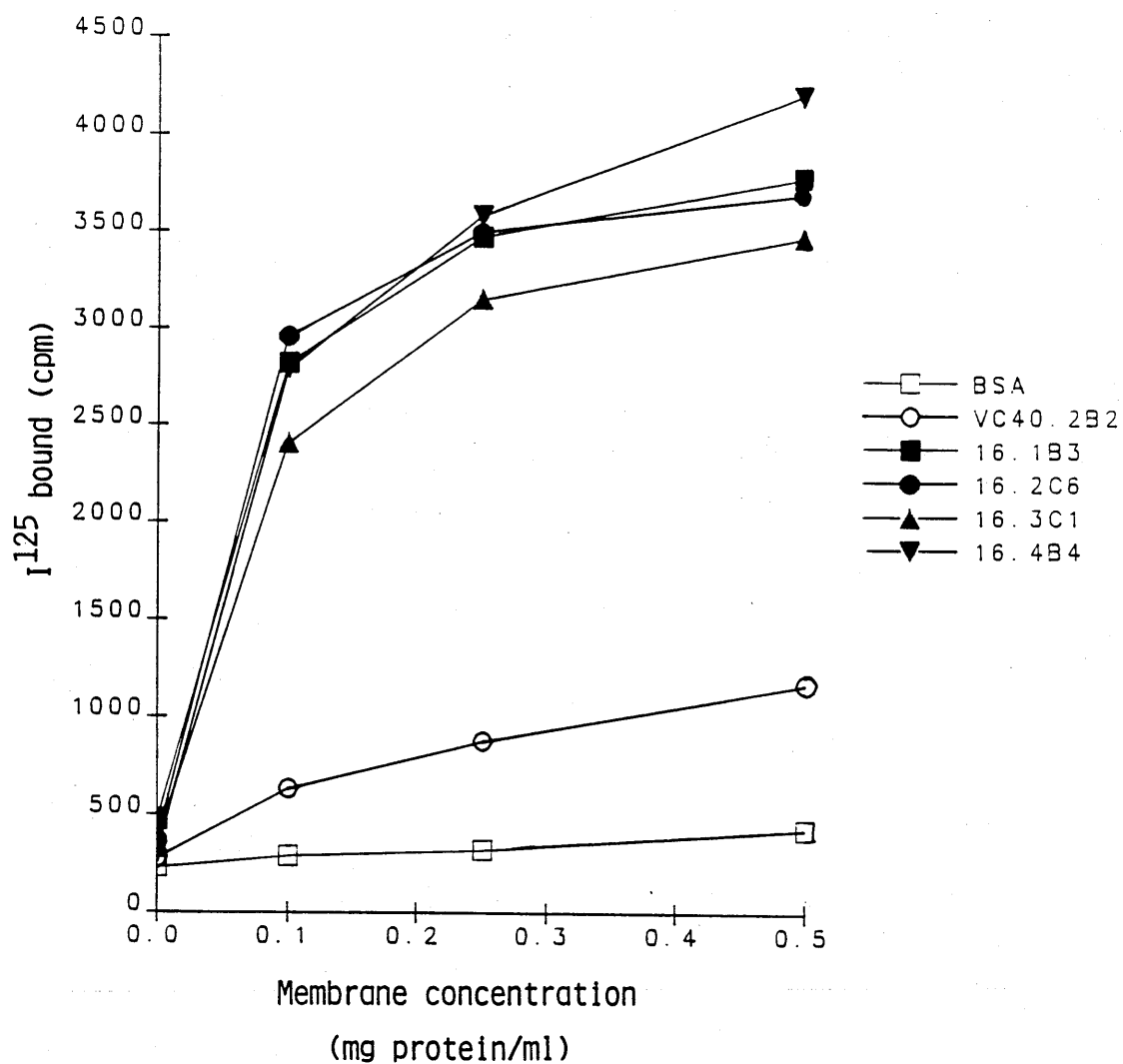


Figure 3.2. Membrane concentration dependence of radioimmunoassay signal for monoclonal antibodies.

Membrane preparation was diluted to 0.1, 0.25 and 0.5 mg protein/ml in PBS-azide-0.1% BSA, and used as substrate in the radioimmunoassay using the undiluted hybridoma supernatants indicated.

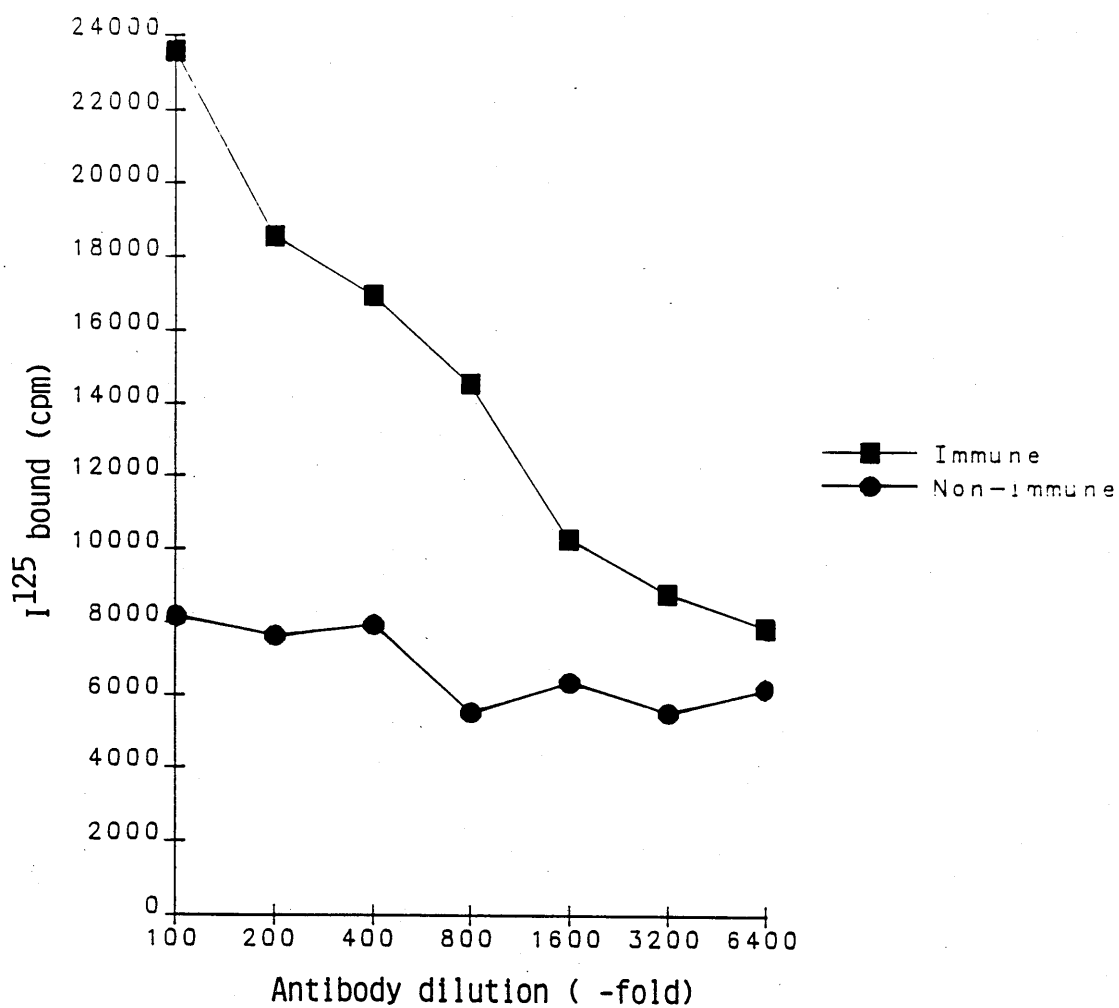


Figure 3.3. Polyclonal antibody concentration dependence of radioimmunoassay signal.

Polyclonal immune and non-immune antisera were diluted 100-fold with PBS-A-BSA, then by two-fold dilutions to 6400-fold. Membrane concentration was constant at 0.5 mg protein/ml.

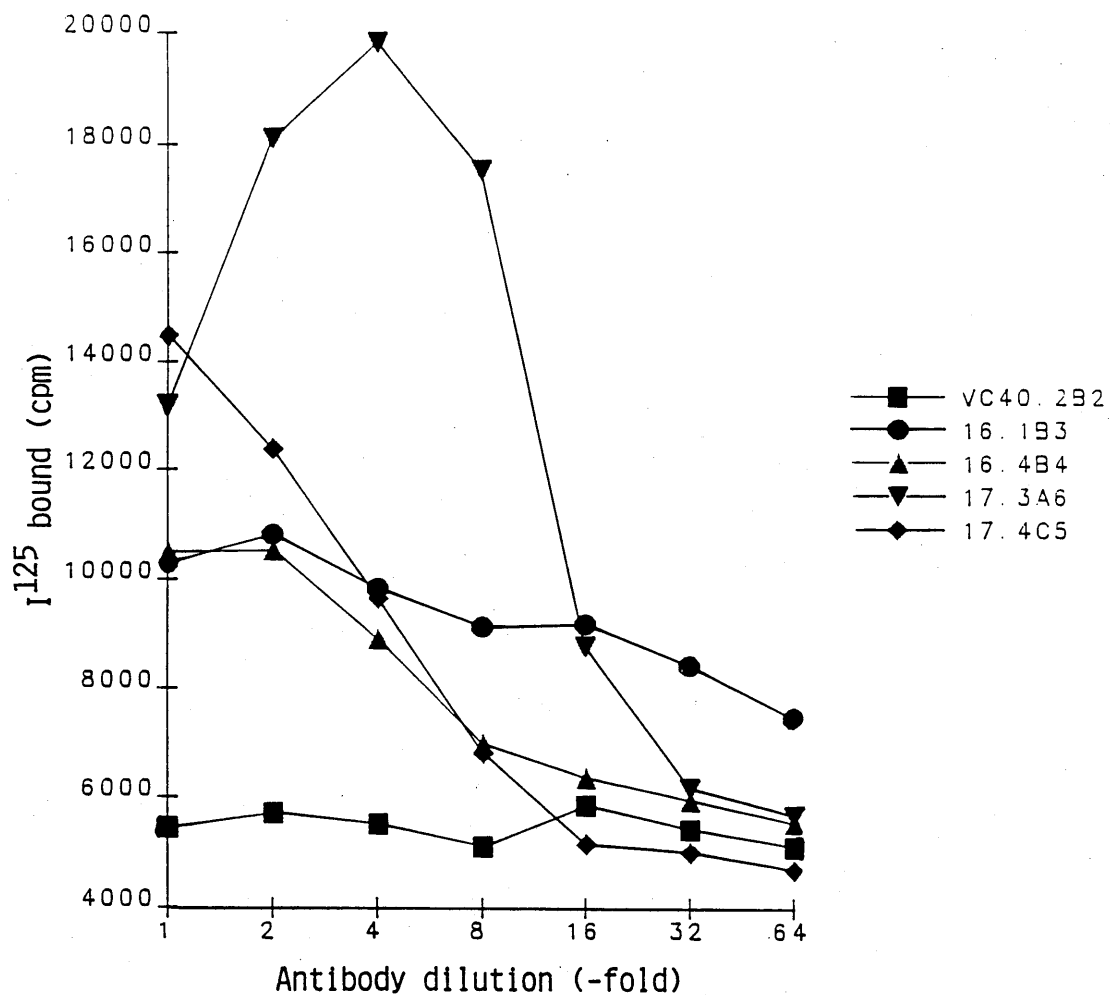


Figure 3.4. Monoclonal antibody concentration dependence of radioimmunoassay signal.

The hybridoma supernatants indicated were subjected to two-fold serial dilutions up to 64-fold in PBS-A-BSA. Membrane concentration was equivalent to 0.5 mg protein/ml.

## Production and cloning of hybridomas.

Three fusions produced large numbers of secreting hybridoma cell lines. Table 3.2 summarises the results for these fusions under four criteria:

- 1) The number of wells in which hybridomas grew.
- 2) The number of hybridomas giving signal:noise ratios at the time of picking to 6 cm petri dishes of 2.0 or above.
- 3) The number producing antibody secreting subclones after one round of limiting dilution.
- 4) Clones producing non-secreting clones only upon limiting dilution.
- 5) The number for which a selected subclone produced a supernatant with a signal:noise ratio above 2.0 when grown in 100 ml of HT medium.

The 14 clones for which subclones grew stably in 100 ml of HT medium were selected for further studies and development of new procedures. A further 11 clones which produced secreting hybrids after limiting dilution failed to secrete antibody upon growth in 100 ml of HT medium. Supernatants from 6 ml cultures of these clones, which contained secreted antibody were used in some subsequent procedures after these had been optimised.

Clone designations and signal:noise ratios for each group are summarised in Table 3.3.

Table 3.2. Results of productive fusions and limiting dilutions.

Fusion number	16	17	23
Total wells plated	96	96	96
Wells containing hybrids	62	86	28
Hybrids with signal:noise >2.0 in multiwell plates	27	52	5
Hybrids producing positive clones upon limiting dilution	9	17	4
Hybrids producing negative clones only upon limiting dilution	2	7	1
Hybrids growing stably in 100 ml of HT medium	5	7	2

Table 3.3. Clonal hybridoma cell lines.

Secreting in 100 ml cultures		Not secreting in 100 ml cultures	
Clone	Signal:noise	Clone	Signal:noise
16.1B3	4.2	16.2D4	2.4
16.2C6	3.7	16.2D5	1.9
16.3C1	3.7	16.4A4	2.5
16.4A6	3.4	17.1A5	2.0
16.4B4	4.6	17.1C6	2.1
17.2B4	3.6	17.2C4	2.0
17.3A3	2.5	17.2D6	2.3
17.3A6	4.2	17.3B6	3.1
17.3B4	3.5	17.4A1	3.0
17.3C4	3.0	23.1B5	2.1
17.4B5	3.6	23.3A1	2.3
17.4C5	4.4		
23.1D5	5.2		
23.2D3	3.4		

## Cross-species reactivity.

Cross-species reactivity was measured by radioimmunoassay using membranes prepared from a variety of plant tissue cultures available in the laboratory. Results were compared to those obtained using N. glutinosa total cellular membrane preparations. Protoplasts were prepared from N. glutinosa suspension culture cells as described in Chapter 2, and washed into homogenisation buffer, in which they were disrupted using 20 strokes of a glass-glass homogeniser. The membrane was prepared by centrifugation at 1000  $g_{av}$  then at 100 000  $g_{av}$  as described in Chapter 2, and dispersed in PBS by homogenisation. Membrane was also prepared from N. plumbaginifolia suspension culture, Petunia hybrida callus, Glycine max and Phaseolus vulgaris suspension cultures and Triticum aestivum callus as described. Protein content was determined by the method of Lowry *et al.* (1951) and membrane preparations diluted to 0.5 mg/ml in PBS for radioimmunoassay. Results are expressed in Table 3.4 on a scale of -(no binding) to +++(strong binding).

As may be seen from Table 3.4, there was a high degree of cross-species reactivity for all the clones tested. None differentiated between the different Solanaceae tested, whilst many also cross-reacted with the Leguminaceae in the test. Other clones (16.1B3, 16.2C6, 16.3C1, 16.4B4) showed some differentiation between the Solanaceae and Leguminaceae, whilst others (17.3D4, 17.4B5, 17.4C5) showed background binding to the Leguminaceae.

The pattern of cross-reactivity with wheat membranes was similar to that on the Leguminaceae, with clones 16.1B3, 16.2C6, 16.3C1, 16.4A6, 16.4B4, 17.4B5 and 23.1D5 showing a moderate degree of cross-reaction, and 17.3A6, 17.3B4, 17.3D4 and 17.4C5 showing no binding to the membranes.

Table 3.4. Comparative radioimmunoassay on membrane preparations from tissue cultures of a variety of plant species.

Clone	<u>N. glutinosa</u> protoplasts	<u>N. plumbaginifolia</u> suspension callus	<u>P. hybrida</u> callus	<u>G. max</u> suspension	<u>P. vulgaris</u> suspension	<u>T. aestivum</u> callus
16.1B3	+++	+++	+++	++	+	++
16.2C6	+++	+++	+++	++	++	++
16.3C1	+++	+++	+++	+++	++	++
16.4A6	+++	+++	+++	++	+++	++
16.4B4	+++	+++	+++	4	+	++
17.3A3	n.t.	n.t.	n.t.	n.t.	n.t.	+++
17.3A6	n.t.	n.t.	n.t.	n.t.	n.t.	+/-
17.3B4	-	+++	+++	+++	++	+/-
17.3D4	+++	+++	+++	+/-	+/-	+/-
17.4B5	+/-	+++	+++	+/-	+/-	+
17.4C5	+++	+++	++	+/-	+/-	+/-
23.1D5	+++	+++	+++	+++	+++	+
23.2D3	+	+++	+++	+++	+++	++

Key: - +/- + ++ +++ weakest to strongest binding  
n.t. not tested



## DISCUSSION.

### General strategy of hybridoma production.

Köhler and Milstein (1975) described the production by fusion of splenic lymphocytes and myeloma cells, of immortalised cell lines which continue to secrete antibody of specificity determined by the lymphocyte parent. Production of these hybridomas relies on two previous discoveries, namely techniques to fuse cells with high frequency, and the ability to select individual fusants between different cell types.

The selection of hybridomas has the advantage that unfused splenic lymphocytes are unable to proliferate in culture, and so need not be selected against. Unfused myeloma cells were selected against by the HAT selection procedure of Littlefield (1964). Many myeloma lines used for fusion are deficient in hypoxanthine-guanine phosphoribosyl transferase activity. S194 was selected by 5-bromodeoxyuridine resistance for thymidine kinase deficiency. Since S194 cells are unable to salvage thymidine, they must produce it de novo. Aminopterin is a dihydrofolate analogue which inhibits dihydrofolate reductase, thus lowering the cellular THF level and suppressing de novo synthesis of nucleotides when present in the growth medium. Thus in HAT medium, S194 myeloma cells are unable to salvage or synthesise thymidine, cannot synthesise DNA, and die.

Fusion with a splenic lymphocyte provides a functional copy of the thymidine kinase gene, and so complements the deficiency in the S194 myeloma cell, enabling the hybrid to grow. Thus hybrids between splenic lymphocytes and myeloma cells are the only lymphoid cells which will proliferate in culture. Although unfused fibroblasts from the spleen may also grow to some extent, these will be outgrown by hybridoma clones. It is important to select in BUdR immediately prior to fusion to eliminate revertants to  $TK^+$  phenotype which occur spontaneously with a low frequency (ca.  $10^{-6}$ ).

Fusion of cell lines initially used Sendai virus, which was subsequently replaced with polyethyleneglycol as the agent of choice (Galfre et al., 1977). Fusion is apparently effected by increase in the hydrophobicity of the medium, resulting in close apposition of plasmalemmae of adjacent cells which in some cases become contiguous, resulting in cell fusion. Fusion initially produces heterokaryons, with common cytoplasm, but separate nuclei. A fraction of heterokaryons will form synkaryons in which the nuclei fuse to generate cells with single hyperdiploid nuclei. This system has been used extensively to study the control of expression of differentiated function in animal cells (Harris, 1971; Weiss and Chaplain, 1968). It is usual in such fusions for the expression of the differentiated function to be suppressed in the hybrid, unless the fused cells are of the same developmental origin (Jones et al., 1976). This is the case in the fusion of antibody producing and non-antibody producing cell lines. Cotton and Milstein (1973) demonstrated, however, that fusion between two antibody producing myeloma lines resulted in hybrids secreting antibodies of both parental types. Initially, the myeloma lines used in hybridoma production secreted antibodies, so that hybridomas derived from them secreted antibodies of two specificities, but subsequently myeloma lines have been derived which no longer secrete antibody, but still support antibody production from the immunoglobulin genes of the lymphocyte parent.

An important feature of hyperdiploid cell lines is their tendency to lose chromosomes. This occurs in a random manner and at different rates for different hybrid cell lines, until a stable karyotype is produced. This is a source of loss of antibody secretion from hybridomas. It has been shown (Hengartner et al., 1980) that loss of antibody secretion is usually by loss of heavy chain, rather than light chain synthesis, since the genes for the two chains reside on different chromosomes (heavy chain: chromosome 12; light chain kappa: chromosome 6, lambda: chromosome 16). The thymidine kinase gene is on chromosome 11, so hybrids can lose secretion of antibody through loss of chromosomes 6, 12 or 16 of the lymphocyte parent without loss of ability to grow under HAT selective conditions. Alternatively, loss of

chromosome 11 from the lymphocyte parent will result in loss of thymidine kinase activity and ability to grow under HAT selection without loss of potential to secrete antibody. Taggart and Samloff (1983) have devised a selection scheme in which the selectable marker is on chromosome 12, and so is linked to the heavy chain gene, resulting in simultaneous loss of the two potentialities, and elimination of non-producing segregants. A similar selection strategy has been used in other systems (Deisseroth et al., 1980). Such selection methods will increase the number of secreting hybridoma lines generated, and the ease with which they may be stabilised by cloning relative to the classical HAT selection.

Selection with aminopterin cannot be maintained indefinitely, as reduction of tetrahydrofolate levels affects other cellular processes. Thus the cultures must be transferred to HT medium, in which selective pressure is no longer exerted. Overgrowth of the secreting clone by one or more faster growing non-secreting clones in the same well can also cause loss of antibody secretion. This can be reduced by plating the cell suspension after fusion into a greater number of wells, but this becomes counter-productive, since the number of clones which will grow decreases as the density of cells decreases (De Blas et al., 1983). To counter both these effects, it is necessary to produce stable clonal cell lines.

Cloning by the method of limiting dilution was used in preference to cloning in soft agar. Certain myeloma cell lines will divide in culture to produce colonies when they are the only cells present. Such is the case for S194 and many of the hybridomas derived from it. Since it often occurs that few colonies grow at one cell/well, clones were diluted to 2 and 10 cells/well. Clones which produce no colonies upon dilution to 2 cells/well often will do so at the lesser dilution with continued antibody production. As can be seen from Table 3.2, failure to grow under cloning conditions and loss of secretion are major sources of loss of useful hybridomas. It is possible that the former problem might be overcome for some clones by the inclusion of feeder cells, eg: thymocytes (Lernhardt et al., 1978), non-immune spleen

cells (Galfre et al., 1977), irradiated fibroblasts (Brodsky et al., 1979) or peritoneal macrophages (Fazekas de St. Groth and Scheidegger, 1980; Boss, 1984). These may provide cell-cell contact and other factors which will promote growth of the hybridoma clone whilst themselves not dividing and eventually dying in culture. Their use was not attempted, but might have been useful for some recalcitrant clones.

Cryopreservation is a vital technique to preserve these vigorously growing and potentially genetically unstable cell lines in a state where continued antibody production can be guaranteed. The process is highly efficient when used on vigorous clones grown to high density, and rarely fails to regenerate viable clones on thawing. Some hybridomas, however, will not grow efficiently to a high enough density to be frozen, and these occasionally will not survive cryopreservation. Sometimes a further cloning will isolate more vigorous subclones which may be cryopreserved more efficiently.

#### Immunisation schedule.

The immunisation schedule for the mice used in production of hybridomas is a critical factor which decides the ultimate level of success of the experiment, but is unfortunately also extremely difficult to optimise. A number of factors influence the efficiency of the immunisation, including the response of the animal to the antigen, which due to epigenetic factors in determination of antibody repertoire and response will differ even between individual animals of an inbred strain (see Chapter 9). In addition, the nature of the antigen will influence the magnitude of the response and the type of cell responding. In a given preparation a few epitopes are usually immunodominant, so that the library of hybridomas produced will be biased towards production of a disproportionate number of clones secreting antibody against them (Chapter 9). In addition, the number of immunisations, their route and timing will modulate the immune response. In these experiments, the schedule used for mice 16 and 17 was an adaptation of that used by for immunisation with

erythroleukaemia cells (Trowbridge et al., 1982). The repeated intraperitoneal immunisations afford chronic stimulation of the immune system, which increases the number of active B cells secreting antibody against the immunogen, and also selects for those producing antibodies of higher affinity. The final intravenous immunisation causes the immune system to be challenged with a large amount of antigen, which should cause a high rate of proliferation of clones directed against it (Stahli et al., 1980). The rate of proliferation is expected to peak 3-4 days after immunisation, at which time the fusion is performed (Anderson and Melchers, 1978). From Table 3.2, it may be concluded that this strategy proved quite successful, whilst that used for mouse 23 was less so. In this case it was hoped that resting the mouse for 4 weeks before the final immunisation would cause the mouse to launch a secondary response to the immunogen, of greater magnitude than the initial one. Technical as well as immunological factors could have contributed to the poorer yield of clones, however, and the lack of an intravenous immunisation may have resulted in less intense stimulation of the immune system. Conclusions based on such a small sample are not statistically valid, however.

#### Radioimmunoassay.

The radioimmunoassay was found to be a rapid and reproducible method of detecting antibody binding to the immunogen and was used to screen all hybridomas through growth, cloning and expansion processes. Results for individual clones were reproducible both within and between assays-the latter subject to some provisos. These are due to the lability of the iodinated antibody, which is subject to degradation of activity from three sources (Bolton, 1977).  $I^{125}$  decays with a half-life of 60 days, so the specific radioactivity of the second antibody decreases significantly over time, but this would be expected to alter test and control samples to the same extent, resulting in no change in the signal:noise ratio. Radiation emitted by the decay of the  $I^{125}$  can cause denaturation of the antibody in the sample, and freezing and thawing of the solution will result in aggregation and denaturation of the antibody. Aggregated and denatured

material in a sample will adsorb non-specifically to substrate and wells in the radioimmunoassay, increasing background whilst the specific signal decreases, resulting in a decrease in signal:noise ratio for positive clones. Over a period of 2-3 weeks this has little effect, but was found to be a significant problem in interpreting results 3-4 weeks after iodination. Second antibody was iodinated every 4 weeks using a fresh batch of  $\text{NaI}^{125}$ , and non-routine assays (eg: cross-species reactivity) were performed during the first week after iodination to maximise sensitivity. A control of known positive binding was included in all assays to give an indication of the expected magnitude of a positive response. Despite these considerations, problems with the radioimmunoassay have led to its replacement with an ELISA procedure, which gives more reproducible results, is performed faster and does not require work with radioisotopes.

The comparison of signal:noise ratios for individual hybrids at the time of picking to 6 cm plates to that at the time of freezing gave an indication of the clones which were likely to continue secretion of antibody through the cloning step. Certain hybrids showed a dramatic decrease in signal:noise between these two steps and were not selected for cloning. Others showed a slight decrease, and a number of these produced secreting clones after limiting dilution cloning, despite the fact that the detection of antibody does not necessarily indicate that the cells which secreted it are still extant. Other hybrids showed little change or an increase in signal:noise. In general, these hybrids produced a number of clones which secreted antibody through limiting dilution. For many hybrids, it proved simple to determine if secretion persisted through cloning, since non-secreting clones showed a signal:noise ratio of around 1 whilst those which continued to secrete antibody showed a signal:noise comparable to that of the initial culture.

## Cross-species reactivity.

Broad cross-species reactivity was observed for all the clones tested. All cross-reacted with all Solanaceae tested and cross-reaction was also seen for many clones with Leguminaceae and the monocot wheat, indicating conservation of the epitopes recognised. This may be due to their being in highly conserved protein sequences, or carbohydrate moieties, the latter since they show more limited potential for variability (see Chapter 9). Broad cross-species reactivity has been demonstrated for other monoclonal antibodies. For example, Dawson *et al.* (1985) have demonstrated that a monoclonal antibody raised by Pruss *et al.* (1981) against glial fibrillary intermediate filament proteins cross-reacts with a protein present in the cytoskeleton of higher plant cells. The differences in species range exhibited for epitopes recognised by different antibodies suggests that a variety of epitopes are recognised and that more than one specificity is exhibited in the hybridoma library.

The test with N. glutinosa protoplast membranes suggested that clones 17.3B4, 17.4B5 and 23.2D3 might bind to determinants on the cell wall, or that were otherwise removed by protoplasting, since the amount of antibody bound was decreased by enzymatic digestion of the cell wall. It would be expected that this treatment would not alter the binding to epitopes expressed within the cell or those on the plasmalemma providing they are not susceptible to digestion by protoplasting enzymes. This hypothesis was tested using the procedures described in the next chapter.



PLANT PLASMALEMMA STRUCTURE:  
AN IMMUNOLOGICAL APPROACH

PAUL MICHAEL NORMAN, PhD

JULY 1986

PART 2 OF 3

CHAPTER FOUR.

DETERMINATION OF SITES OF ANTIBODY BINDING.



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## INTRODUCTION.

In the previous chapter, the production of a library of hybridomas secreting monoclonal antibodies directed against total membranes from N. glutinosa suspension culture cells was described. It is expected that such a library will contain antibodies directed against a variety of antigens from the membrane preparation. Suggestive evidence that this is the case is provided by the cross-species reactivity of the antibodies, which differs for different clones. In this chapter, the heterogeneity of the hybridoma population is addressed with respect to sites of antibody binding, as indicated by two techniques which provide topological information. Immunofluorescence microscopy provides a method of direct visualisation of sites of antibody binding in living or fixed cells and tissues. The technique is here applied to fixed and unfixed N. glutinosa cells, and protoplasts derived from them.

Immunofluorescence microscopy is constrained by the need to examine a small field of protoplasts, and without a microfluorimeter attachment cannot generate a quantitative measurement of fluorescence. Flow cytometry is a useful complementary procedure since the instrument has the capacity to make quantitative fluorescence measurements on a large number of individual particles. It will not, however, discriminate between intact protoplasts and debris. Thus flow cytometric measurements require a protoplast population which has previously been characterised as having a high content of intact protoplasts, although data can be processed to remove the component due to small particles.

Thus the combination of immunofluorescence microscopy and flow cytometry provide complementary information as to the sites of antibody binding, and the characteristics of the population showing such binding. The data from these assays provide direct evidence that the hybridoma library contains a population of clones secreting monoclonal antibodies directed against epitopes exhibited on the plasmalemma of N. glutinosa cells, another which secretes antibodies

against epitopes on the cell wall, and suggests that a number of clones secrete antibodies which recognise epitopes expressed within the cell. The three classes are non-overlapping.

## MATERIALS AND METHODS.

Immunofluorescence microscopy on fixed protoplasts and cells in a saline osmoticum.

Protoplasts were prepared as described in Chapter 2, using 50 ml of 1% cellulase Onozuka RS and 0.1% pectolyase Y23 in 2.5% KCl, 1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1% MES, 0.1% BSA, pH 5.5. After 90 min digestion, they were filtered through absorbant cotton wool, and collected by centrifugation for 3 min at 100  $g_{\text{av}}$ . The supernatant was aspirated and the protoplasts resuspended in 2.5% KCl, 1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2% HEPES, pH 7.2 (saline osmoticum) with which they were washed a second time. A 5% formaldehyde solution in saline osmoticum was freshly prepared by depolymerisation of paraformaldehyde at pH 11-12, and titration back to pH 7.0-7.5. Protoplasts were fixed in this solution with occasional gentle agitation for 10 min, after which they were washed once before resuspension and incubation for 15 min at 4°C in 10 ml of saline osmoticum containing 1 mg/ml goat gammaglobulins (US Biochemical Corp, Cleveland, Ohio). All subsequent steps were performed at 4°C. Ca.  $10^6$  protoplasts were aliquotted into an osmotically balanced hybridoma supernatant. This consisted of 0.8 ml of supernatant, to which 0.2 ml of 5-fold concentrated osmoticum had been added, to render the osmotic potential of the solution similar to that of the saline osmoticum. The protoplasts were incubated in this solution for 45 min, with occasional gentle agitation, then washed twice in saline osmoticum with 1 mg/ml BSA and 0.1 mg/ml goat gammaglobulins added. The protoplasts were resuspended in 500  $\mu\text{l}$  of the above solution containing a 1:10 dilution of fluorescein-labelled goat anti-mouse Ig's (HyClone, Logan, Utah), and incubated with occasional gentle agitation for 45 min. They were washed three times in osmoticum-BSA-gammaglobulins, resuspended, and drops of suspension placed on cover slips. Fluorescence was viewed using a Nikon Diaphot (Nippon Kogaku K.K., Tokyo, Japan) inverted microscope with epifluorescence attachment, x20 and x40 fluorescence objectives and B filter block (excitation wavelength 460-485 nm, and barrier filter transparent to wavelengths greater than 515 nm).

In some cases, protoplasts were also stained with calcofluor white M2R (Polysciences, Warrington, Pennsylvania), as described by Hahne et al. (1983). A solution of the dye was prepared by suspending 1 mg in 10 ml of saline osmoticum. This was left overnight at 4°C, and undissolved calcofluor removed by filtration through a 0.2 µm pore size nitrocellulose membrane. The stain was applied to the protoplasts in the first wash after incubation with fluorescein-labelled antibody, and unbound stain removed by the subsequent washes. Calcofluor fluorescence was observed using a UV filter block (excitation wavelength 330-380 nm, transparent to wavelengths greater than 420 nm).

Immunofluorescence staining of cells was carried out as described above, except that the initial protoplasting was omitted. The cells were collected and washed with saline osmoticum prior to fixation. It was found that the fixation step could be omitted without detriment to the procedure.

Immunofluorescence microscopy on unfixed protoplasts in a sorbitol osmoticum.

Protoplasts were also prepared and immunofluorescent staining effected in a medium in which the osmoticum was sorbitol. The protoplasting was performed as described in Chapter 2, and the immunofluorescence staining as described above, except that the osmoticum was composed of 0.4 M sorbitol, 0.2% HEPES, pH 7.2 (sorbitol osmoticum), hybridoma supernatants were osmotically balanced with 5-fold concentrated sorbitol osmoticum, and the fixation step was omitted. BSA, goat gammaglobulins and fluorescein-conjugated antibody were added to the medium at the same concentrations as described above.

## Flow cytometry.

Flow cytometry was performed at the National Flow Cytometry Resource, Los Alamos, New Mexico. The FACS II optical bench (Becton Dickinson, Mountain View, California) was modified to the specifications of, and operated by Dr. Jim Jett (Los Alamos National Laboratory), as described (Jett and Alexander, 1985).

Protoplasts prepared in sorbitol osmoticum as described above were labelled with hybridoma supernatants diluted 1:1 with sorbitol osmoticum, followed by fluorescein conjugated goat anti-mouse antibody. They were examined by fluorescence microscopy to determine the quality of the preparation, and filtered through 66  $\mu$ m mesh nylon filters to remove aggregates which might clog the machine.

Flow cytometry used a 200  $\mu$ m diameter nozzle, and a sheath fluid composed of 3% (w/v) KCl, 1% (w/v)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1% (w/v) MES, pH 5.7. The sheath pressure was 7 psi, and the piezoelectric drive frequency 4.5 kHz. The inline flow resistor of the sample delivery line was bypassed. Fluorescence excitation was at 457.9 nm by a 350 mW argon ion laser (Coherent, Palo Alto, California). Green fluorescence was measured using a 530  $\pm$  15 nm bandpass filter. Signal processing and data acquisition electronics were as described (Griffith et al., 1984).

## RESULTS.

Immunofluorescence staining in saline osmoticum.

A radioimmunoassay was performed on the total cellular membrane preparation in the presence of various osmotica. The results indicated that use of sugar alcohol osmotica might result in rather high levels of non-specific binding of monoclonal antibodies to intact protoplasts in these media, whereas use of osmotica composed of inorganic salts, such as that described by Meyer (1975) might allow greater differentiation of positive from negative binding. Cells could readily be plasmolysed in such osmotica, and protoplasts were rapidly released, but unfortunately were rather unstable when subjected to the repeated changes in medium and centrifugation necessary for indirect immunofluorescent staining. In an attempt to stabilise the protoplasts, a fixation step was included. Glutaraldehyde or the EGS fixative (Willingham, 1980) as modified by del Rio et al. (1983) resulted in high levels of autofluorescence of the protoplasts and could not be used satisfactorily. Formaldehyde did not cause autofluorescence, but did not stabilise the protoplasts as well as the other fixatives, so that they were prone to evacuation, lysis and aggregation during the procedure. Despite these problems, it proved possible to bring intact formaldehyde fixed protoplasts through the procedure, and to demonstrate specific immunofluorescence on the plasmalemma (Fig. 4.1).

Controls used were DMEM-serum, VC40.2B2 (raised against Pseudomonas syringae var. glycinae) and polyclonal non-immune serum. All of these produced a faint fluorescence on intact protoplasts, which was diffuse and not associated with any particular structure, originating mainly from the centre of clumps, which suggested that it was due to insufficient washing out of non-bound antibody. In contrast, polyclonal immune serum and supernatants from a number of clones produced a strong "ring" fluorescence at the periphery of protoplasts, which was continuous around most of them. This is characteristic of binding to the plasmalemma. If the focus was shifted

slightly, it was possible to view the fluorescence on the faces of the cell perpendicular to the viewer. It was found that the fluorescence was somewhat "blotchy" on the surface, being distributed in small lines and speckles which were evenly distributed across the section (results not shown). Of the 14 clones tested, 16.1B3, 16.2C6, 16.3C1, 16.4A6, 16.4B4, 17.3D4 and 17.4B5 showed ring fluorescence, whilst clones 17.2B4, 17.3A3, 17.3A6, 17.3B4, 17.4C5, 23.1D5 and 23.2D3 showed a level and pattern of fluorescence comparable to that of controls. Calcofluor white staining indicated that no residual cell wall material was present on the surface of the protoplasts, since the fluorescence characteristic of dye bound to undigested cell walls was not seen (results not shown).

Immunofluorescence was also performed on intact cells, which had not been subjected to enzymatic digestion (Fig. 4.1). The controls gave a similarly low background fluorescence to that observed on protoplasts. The set of clones secreting antibodies which reacted with the plasmalemma gave background levels of binding to intact cells, as did antibody secreted by the clones 17.3A3, 17.3A6, 17.4C5 and 23.1D5. In contrast, clones 17.3B4 and 23.2D3 produced antibodies which showed strong specific binding to the cell wall of intact cells.

Thus the clones may be grouped into three categories of binding to the protoplasts and cells, as summarised in Table 4.1.



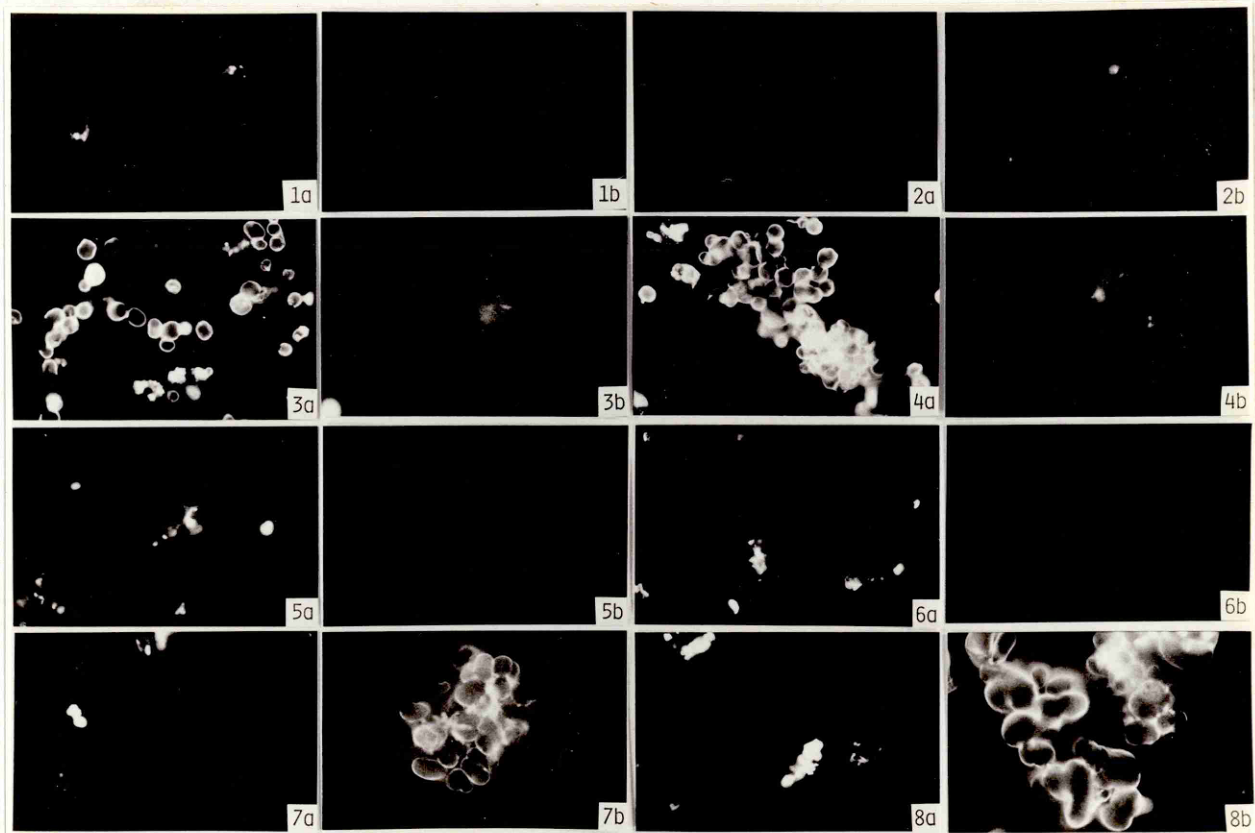


Figure 4.1. Immunofluorescence microscopy of fixed *N. glutinosa* protoplasts and cells.

Substrates were: a) protoplasts; b) cells. Antibodies used were: 1) DMEM; 2) VC40.2B2; 3) 16.2C6; 4) 16.4B4; 5) 17.3A3; 6) 17.3A6; 7) 17.3B4; 8) 23.2D3.

Table 4.1. Results of immunofluorescence assay.

Clone	Binds to:		
	Plasmalemma	Cell walls	Neither
16.1B3	+		
16.2C6	+		
16.3C1	+		
16.4A6	+		
16.4B4	+		
17.2B4			+
17.3A3			+
17.3A6			+
17.3B4		+	
17.3D4	+		
17.4B5	+		
17.4C5			+
23.1D5			+
23.2D3		+	

Immunofluorescence staining of unfixed protoplasts in sorbitol osmoticum.

It was found that use of a sorbitol osmoticum with fixed protoplasts resulted in a high level of non-specific binding of antibody. In contrast, non-fixed protoplasts showed little or no non-specific binding of antibody in sorbitol osmotica. This had three advantages over the use of fixed protoplasts in saline osmoticum, namely a lower level of background fluorescence, increased stability of the protoplasts to the procedure, and preservation of viability. The results of experiments on binding of antibodies to the surface of protoplasts were identical to those found for fixed protoplasts in saline osmoticum, as catalogued in Table 4.1. Use of sorbitol osmoticum with intact cells resulted in high levels of non-specific binding of antibody to the cell wall.

Immunofluorescence staining of leaf protoplasts.

Immunofluorescence assays were also performed on unfixed protoplasts from N. tabacum and P. vulgaris leaves, which were prepared in 0.4 M sucrose osmoticum, as described in Chapter 2, then transferred to 0.4 M sorbitol. Leaf protoplasts showed red autofluorescence of their chlorophyll under fluorescence excitation, which did not interfere with visualisation of the green fluorescence of bound second antibody, since the former was confined to the chloroplasts, whilst the latter was associated with the plasmalemma. N. glutinosa leaf protoplasts were also subjected to immunofluorescence assay, but tended to be smaller and more fragile than those of N. tabacum. The pattern of binding for the set of antibodies on leaf protoplasts from the two species was identical.

The results of the experiments are shown in Table 4.2. The first column of the table indicates the results of immunofluorescence assay on unfixed N. glutinosa suspension culture protoplasts. All clones for which the supernatant gave positive immunofluorescence on these protoplasts also did so on N. tabacum leaf protoplasts. In contrast,

when P. vulgaris leaf protoplasts were used, clones 16.4B4, 17.3D4 and 17.4B5 showed weaker binding than other clones, eg: 16.2C6 and 16.4A6, which showed intensity similar to that on N. glutinosa suspension culture and N. tabacum leaf protoplasts. It was found that upon tenfold dilution of the hybridoma supernatants, 16.4B4 and 17.3D4 showed essentially no binding to P. vulgaris leaf protoplasts, whilst binding to N. glutinosa suspension culture and N. tabacum leaf protoplasts was unaltered. Tenfold dilution of 16.2C6 and 16.4A6 did not result in noticeable decrease in the extent of binding to N. tabacum or P. vulgaris protoplasts (see also Chapter 8).

Table 4.2. Immunofluorescence assay on N. glutinosa suspension culture protoplasts, N. tabacum leaf protoplasts and P. vulgaris leaf protoplasts.

Clone	<u>N. glutinosa</u> suspension	<u>N. tabacum</u> leaf	<u>P. vulgaris</u> leaf
VC 40.2B2	-	-	-
16.1B3	+	+	+
16.2C6	+	+	+
16.3C1	+	+	+
16.4A6	+	+	+
16.4B4	+	+	-
17.2B4	-	-	-
17.3A3	-	-	-
17.3A6	-	-	-
17.3B4	-	-	-
17.3D4	+	+	-
17.4B5	+	+	-
17.4C5	-	-	-
23.1D5	-	-	-
23.2D3	-	-	-

## Flow cytometry.

Figure 4.2 shows the results of flow cytometry of 40 000 particles labelled with different monoclonal antibodies. Channel number as plotted on the X-axes of the graphs is a measure of the relative fluorescence intensity of a particular particle passing through the detection chamber. Results are plotted in Figure 4.2C as the average of 10 channels, and expressed as a percentage of the maximum number of events per 10 channel average. Small angle light scattering was measured simultaneously with fluorescence intensity for each event. Small angle light scattering, which is a measure of particle size, showed bimodal curves, with a component of low light scattering due to debris in the protoplast preparation, and a second of higher light scattering due to protoplasts (Fig 4.2A). The light scattering data were processed to eliminate the component due to debris, and the fluorescence data replotted for the refined data (Fig. 4.2B and C).

The results indicate that there is a clear shift to higher values of the fluorescence intensity curves for the two surface reactive antibodies (16.3C1, 16.4B4) relative to a control using VC40.2B2 and to hybridomas judged by immunofluorescence microscopy to recognise determinants expressed within the cell (17.3A6) or on the cell wall (17.3B4).

Figure 4.2B shows the effect of dilution of a sample of purified 16.4B4 antibody on fluorescence intensity. Decrease in amount of antibody added to a fixed number of protoplasts shifts the fluorescence intensity curves to lower values, despite the presence of a constant amount of fluorescein labelled goat anti-mouse antibody, indicating that surface fluorescence is an antibody specific effect, and not due to non-specific second antibody binding.

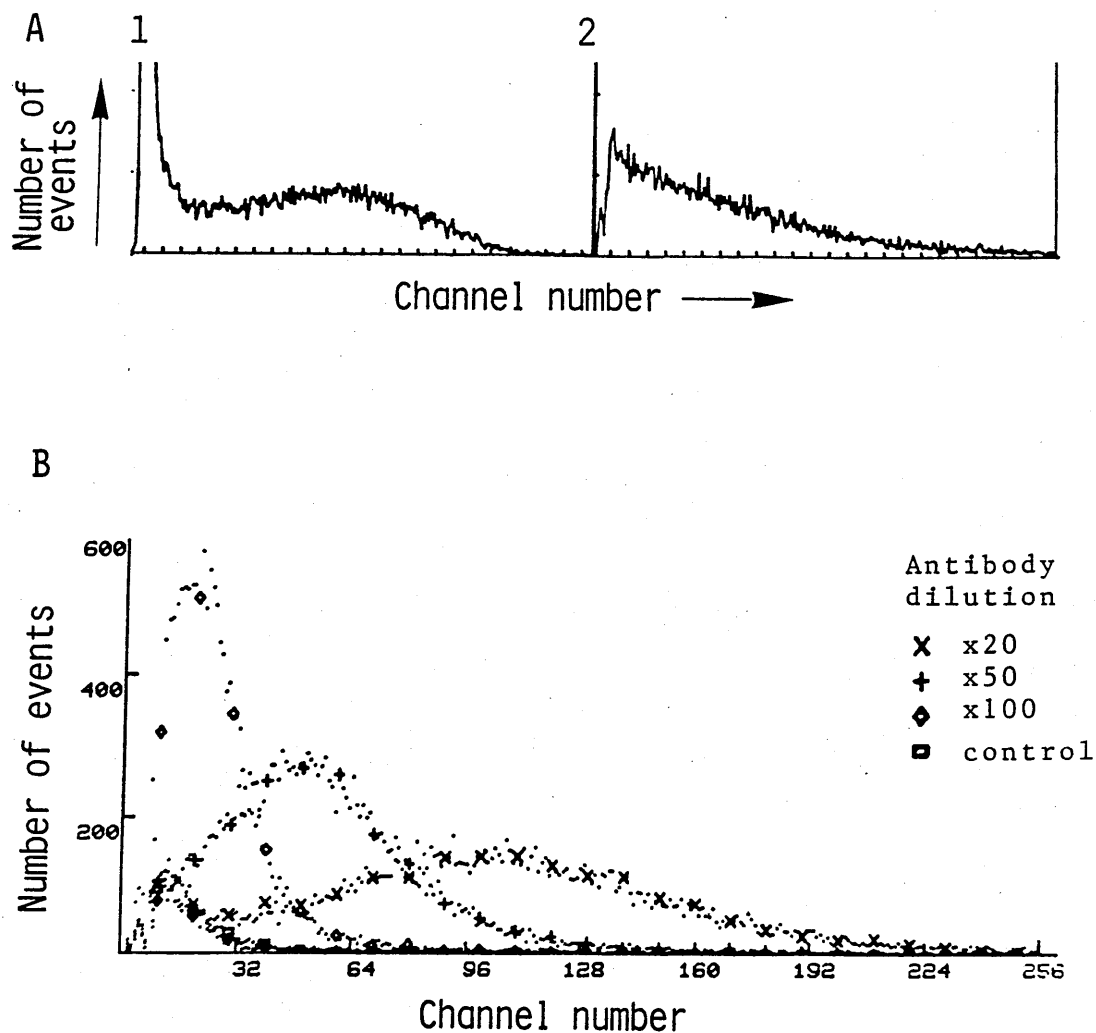
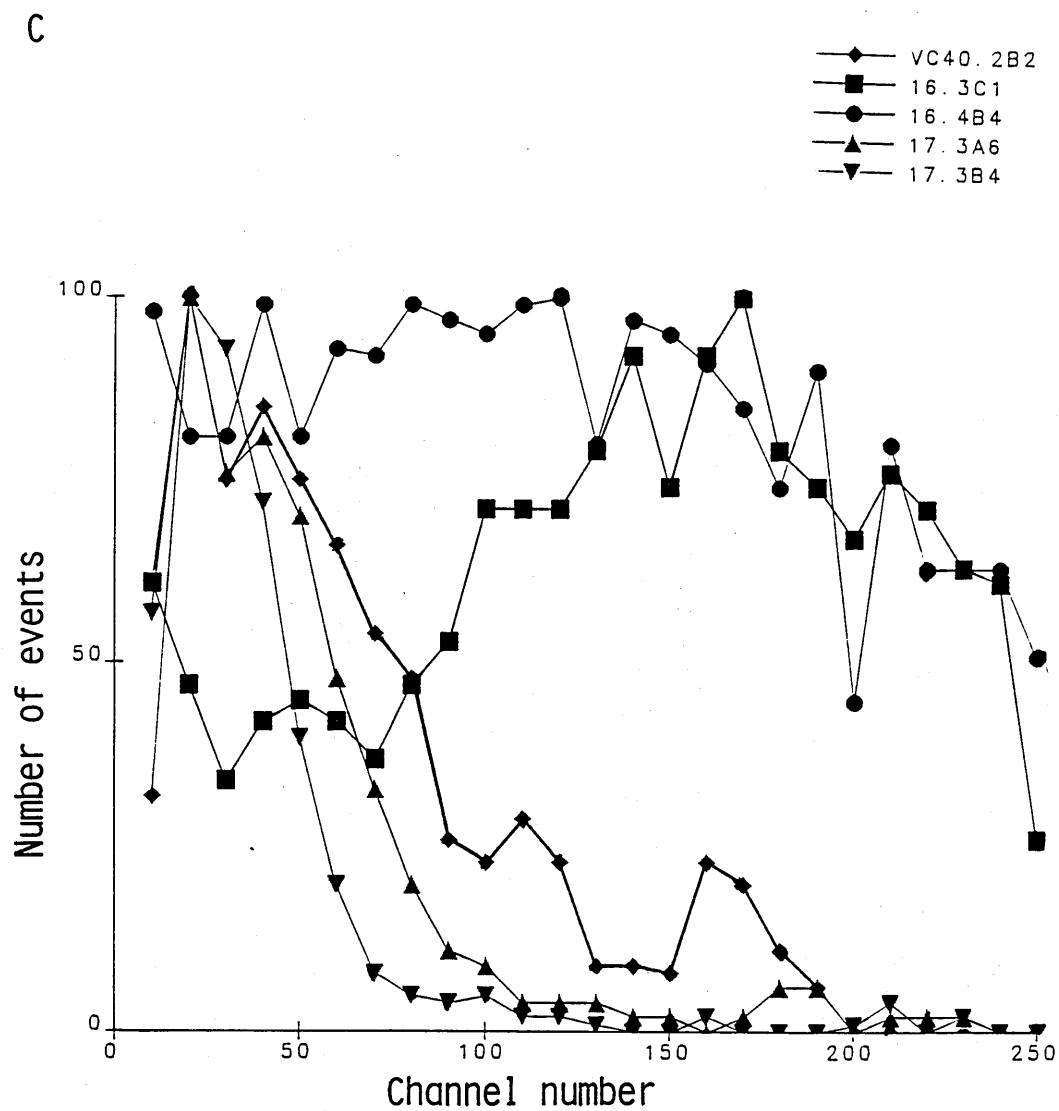


Figure 4.2. Flow cytometry of antibody labelled protoplasts.

A: 1) light scatter and 2) unprocessed fluorescence intensity data for protoplasts labelled with supernatant from hybridoma 16.1B3.

B: Processed fluorescence data for protoplasts labelled with purified 16.4B4 antibody at the dilutions indicated.



C: Processed fluorescence data for protoplasts labelled with the hybridoma supernatants indicated, diluted 1:1 in osmoticum.



## DISCUSSION.

Indirect immunofluorescence microscopy provides a powerful tool, which has been used extensively to visualise sites of antibody binding in both animal and plant cells and tissues. The technique has the advantage of allowing direct visualisation and assignment of sites of antibody binding in living, fixed or sectioned material, but is not as complex a procedure as electron microscopy. In this case, immunofluorescence microscopy has been applied to fixed and unfixed cells and protoplasts from *N. glutinosa* suspension cultures, and to unfixed protoplasts from *N. tabacum* and *P. vulgaris* leaves, to define the sites of binding of antibodies secreted by hybridomas in the library.

There are a number of possible problems in the use of both fixed and unfixed material in immunofluorescence assays. Formaldehyde fixation can result in loss of antigenicity of some epitopes (Altmannsberger *et al.*, 1981), which might present a problem when using a monoclonal antibody, since it recognises only one epitope. In contrast, metabolic processes in unfixed tissues might alter the pattern of antibody binding, for example by patching and endocytosis of the bound antibody. Such processes are well known in mammalian cells (reviewed by Pearse and Bretscher, 1981; Pastan and Willingham, 1981) and have also been demonstrated in plant protoplasts for cationised ferritin (Tanchak *et al.*, 1984), over a period of 10 s-40 min. By keeping the protoplasts on ice through the procedure, such processes should be minimised. That the results were identical using fixed and unfixed protoplasts indicates that possible artifacts from either procedure were not encountered. Whereas the use of fixatives has the potential advantage of stabilising the protoplasts, use of unfixed protoplasts retains viability and allows potential regeneration to callus and plants. The latter advantage has been exploited in a subsequent study (Chapter 8).

Immunofluorescence assay allows the clones to be grouped in three non-overlapping sets, namely those for which the supernatant reacts

with the cell wall (17.3B4, 23.2D3); those which secrete antibody which reacts with the plasmalemma (16.1B3, 16.2C6, 16.3C1, 16.4A6, 16.4B4, 17.3D4 and 17.4B5); and those which secrete antibody which reacts with neither (17.2B4, 17.3A3, 17.3A6, 17.4C5 and 23.1D5). Those reacting with neither plasmalemma nor cell wall are postulated to secrete antibodies directed against internal components of the cell, since they showed binding in radioimmunoassay with total cellular membranes, but not in immunofluorescence assay with intact protoplasts. That they did not show binding in the latter assay also suggests that formaldehyde fixation did not render the protoplasts permeable to antibodies. That there is no residual calcofluor fluorescence, indicates that the cell wall has been completely removed. This contention is supported by data obtained for clones 17.3B4 and 23.2D3 which secrete antibodies which react with intact cells but not with protoplasts.

All clones produced by fusion 16 reacted with the plasmalemma of intact protoplasts, whilst fusion 17 produced hybridomas with a wider variety of specificities, as reflected by immunofluorescence assay. It is perhaps somewhat surprising that 50% of the hybridomas investigated secrete antibodies directed against the exterior face of the plasmalemma, since the total membrane preparation must contain membranes from a number of other organelles. Two possible causes of this suggest themselves. First, this may be a result of the small sample size. It would be necessary to characterise a much larger library of hybridomas to determine if this reflected the true distribution of antigenic material within the cell. Of all hybridomas produced against plant material in the laboratory, a similar proportion react with the plasmalemma as determined by immunofluorescence microscopy. Second, it is possible that the antigens exhibited at the external face of the plasmalemma might be immunodominant. It is known in mammalian cells that the portion of plasmalemma proteins on the external face is usually glycosylated. If this were also the case in plant cells, it might be expected that such glycosyl residues would be immunodominant, particularly if they had a composition unusual in mammals. Such appears to be the case for

monoclonal antibodies raised against Nicotiana glauca style extracts, of which a high proportion recognise beta-D-galactopyranose or alpha-L-arabinofuranose residues of arabinogalactan proteins (Anderson et al., 1984).

The production of two hybrids secreting antibody reacting with the cell wall suggests that the membrane preparation used for immunisation contained cell wall material. This might be expected, since the technique used to prepare the membranes was extremely crude, and shear forces during homogenisation of the cells might result in production of cell wall fragments small enough to remain suspended through the 1000 g centrifugation, but be pelleted in the 100 000 g centrifugation, with the membrane material. That only two clones react with the cell wall might suggest that most of this material had been removed from the membrane preparation. It is significant that none of the antibodies recognising the plasmalemma cross-react with intact cells. IgG molecules have a molecular weight of 150 kd, so presumably the cell wall is impermeable to them, preventing their access to the plasmalemma. This agrees with the results of Carpita et al. (1979), who measured the ability of dextrans and polyethylene glycols of different molecular weights to plasmolyse plant cells. They found that this property was only expressed by dextrans of 6.5 kd or less, and polyethylene glycols of 1.6 kd or less, indicating that only molecules of this size or less can penetrate the cell wall. This is equivalent to a globular protein of ca. 17 kd-considerably less than an immunoglobulin. The exterior of the cell wall must have a composition substantially different from the exterior of the plasmalemma, since there is no cross-reactivity between the two. This suggests that the protoplasts were completely devoid of wall material, corroborating the results of calcofluor white binding.

The results of the radioimmunoassay on N. glutinosa protoplasts reported in Table 3.4 suggested that the sites of antibody binding for different hybridomas might differ. In particular, the decrease in antibody binding for supernatant from hybrids 17.3B4, 17.4B5 and 23.2D3 upon protoplasting suggested that these hybrids might secrete

antibodies which react with components digested by protoplasting enzymes, ie: probably on the cell wall. This hypothesis was confirmed in the case of 17.3B4 and 23.2D3 by the immunofluorescence assay, in which antibodies bound to the cell wall of intact cells, but not to the plasmalemma of protoplasts derived from them.

17.4B5 was the only one of a group of hybridomas secreting antibody which reacts with the plasmalemma of N. glutinosa protoplasts, which showed a decrease in antibody binding upon protoplasting, as measured by radioimmunoassay. This result was not confirmed by the immunofluorescence assay, in which 17.4B5 supernatant gave as strong a fluorescence on the surface of the protoplasts as did other plasmalemma-specific hybridoma supernatants. This may reflect a lower sensitivity of the immunofluorescence assay to changes in epitope availability.

When antibody binding to the cell wall of N. glutinosa suspension culture cells was re-examined after a further 15 months, it was found that neither 17.3B4 or 23.2D3 antibodies bound to the cell wall. During the intervening period, the cell cultures had been maintained at a lower density than at the time when the initial immunofluorescence experiments were performed. When cultures were again maintained at higher density, binding of 17.3B4 but not 23.2D3 antibody reappeared. Thus it would seem that 17.3B4 recognises an epitope which is expressed when the cultures are maintained at high density, but not in less dense cultures. A similar pattern of antigen expression has been demonstrated for epithelial cells, in which a 45 kd cytokeratin was expressed only in dense cultures (Ben-Ze'ev, 1985), and for glucosaminoglycans extracted from mouse fibroblasts in culture (Underhill and Keller, 1976; Cohn et al., 1976; Vannucchi and Chiarugi, 1977).

The results of immunofluorescence assays on N. tabacum and P. vulgaris leaf protoplasts provide confirmation of the sites of antibody binding on plant material of different tissue and species origin, and also an assessment of cross-species reactivity independent

of radioimmunoassay. The subset of clones secreting antibody showing positive surface immunofluorescence on N. glutinosa suspension culture protoplasts also did so on N. tabacum leaf protoplasts, indicating that the antigens recognised are expressed in intact plants and are not unique to suspension cultured cells. This also confirms the radioimmunoassay data showing cross-reactivity with other Solanaceae for these clones. A similar distribution was shown on other Nicotiana species protoplasts for which results are not shown (N. glutinosa leaf, N. plumbaginifolia suspension culture).

The results with P. vulgaris leaf protoplasts also confirm the pattern of cross-reactivity expected from the radioimmunoassay. 16.4B4, 17.3D4 and 17.4B5 showed decreased binding to the plasmalemma relative to 16.2C6, 16.3C1 and 16.4A6. Binding of the former set could be eliminated by suitable dilution of the supernatants, whilst a similar dilution of the latter set did not decrease the level of binding. A similar dilution did not affect the fluorescence intensity for any of the supernatants when immunofluorescence was applied to N. glutinosa suspension culture protoplasts.

Thus at appropriate dilution, certain antibodies will distinguish between protoplasts of different species, indicating that the antigenic determinants recognised differ between species, although the tendency to cross-reactivity at higher concentrations suggests that protoplasts of Leguminaceae exhibit closely related epitopes. In contrast, a second group of clones secretes antibodies which recognise epitopes of more general species distribution.

Flow cytometry confirms that the surface fluorescence on N. glutinosa protoplasts is monoclonal antibody specific. A similar fluorescence intensity curve is seen for the cell wall reactive clone 17.3B4 to that for VC40.2B2. This indicates that cell wall expressed epitopes are not available for antibody binding, ie: that protoplasting is complete, and that the plasmalemma does not express significant levels of the wall component recognised by 17.4B4. Likewise, the fluorescence curve for 17.3A6 is similar to that of

VC40.2B2, indicating that the epitope recognised by this antibody, which is postulated to be expressed within the cell, is unavailable for antibody binding, ie: that the protoplasts are not permeable to antibody.

The results for 16.3C1 and 16.4B4 thus confirm that these antibodies recognise epitopes expressed on the surface of most protoplasts in a population. The curve for 16.4B4 shows rather broad distribution of fluorescence intensity, suggesting some heterogeneity in antibody binding properties of the population. This may be due to protoplast breakage or the presence of more debris of in this than in other samples, however, since the results using purified antibody at low dilution (Fig. 4.2B) show a clear shift to higher fluorescence intensities relative to the higher antibody dilutions, and to the control, and approximately Gaussian shape.

That the fluorescence intensity curves are quite broad is probably related to the heterogeneity in size of the protoplasts. The flow cytometer measures the total fluorescence intensity of the entire particle, which will increase as the square of the particle diameter, for two particles of the same fluorescence intensity per unit surface area. That there is considerable heterogeneity in particle size is indicated by the light scattering curve of particles passing through the detection system (Fig. 4.2A). It is not unexpected that the protoplast size varies greatly in a population, since considerable size variation is exhibited even in a population of synchronised mammalian cells, whereas the *N. glutinosa* cultures have not been selected for uniform growth characteristics in culture, or rendered synchronous. The results suggest that both antibodies 16.3C1 and 16.4B4 recognise uniformly all protoplasts in a population, and probably do not selectively label a subset of protoplasts.

That by the criteria of surface immunofluorescence and flow cytometry a large proportion of the clones raised recognise antigens displayed on the external face of the plasmalemma indicates that this face does exhibit antigenic material, which might indicate the

presence of some plasmalemma structure, such as glycosylation. The possibility that some of the antibodies recognise membrane-bound proteins or glycoproteins is addressed in the following chapter by the use of techniques allowing detection of protein antigens.

CHAPTER FIVE.

DETECTION OF PROTEIN ANTIGENS BY WESTERN BLOTTING  
AND IMMUNOPRECIPITATION.



## INTRODUCTION.

That hybridomas from the library secrete antibodies reacting with a crude membrane preparation from *N. glutinosa* cells was demonstrated in Chapter 3. That these antibodies differ in their subcellular binding sites was shown in the previous chapter. Here, the nature of the antigens which are recognised by individual antibodies is investigated.

It is possible to raise antibodies reactive with a vast number of different antigens, ranging from metal ions to molecules the size of bacterial envelope polysaccharides. It is thus possible that the hybridomas secrete antibodies recognising any of a variety of membrane associated molecules.

Here, using two techniques, the possibility is addressed that certain of the antibodies raised in the current investigation recognise protein or glycoprotein antigens. Western blotting (Towbin *et al.*, 1979) of proteins to nitrocellulose filters, followed by the probing of the filters with antibodies is a powerful method of detection of reactivity with such antigens, but is subject to a number of theoretical limitations as to the nature of epitopes which will be detected. That these limitations are indeed encountered is demonstrated by the detection of a second set of anti-protein reactive antibodies by *in vivo* labelling with radioactive amino acids, followed by immunoprecipitation from extracts of plant cells in non-denaturing detergent solution. This technique also imposes limitations on the epitopes which will be detected. Use of these two methods of analysis therefore isolates a broad range of antibodies reactive against proteins or glycoproteins. Combined with the immunofluorescence data of Chapter 4, the present results illuminate the nature of cell surface expressed proteins.

## MATERIALS AND METHODS.

Western Blotting.

Preparation of cell extracts.

Western blotting was performed using extracts of *N. glutinosa* suspension culture cells in sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, composed of 10% (v/v) glycerol, 5% (v/v) beta-mercaptoethanol, 2% (w/v) sodium dodecylsulphate, 0.005% (w/v) bromophenol blue, 80 mM Tris, pH 6.8.

Cells were harvested from actively growing *N. glutinosa* suspension cultures, 4-7 days after subculture, by collection on miracloth. The cells were washed briefly with distilled water, then ground at room temperature using a pestle and mortar in 1 ml/g fresh weight of SDS-PAGE sample buffer, for 3-5 min. The homogenate was placed in a boiling water bath for 5-10 min, and undissolved material pelleted at 100 000 g<sub>av</sub> for 1 h at 25°C. The supernatant constituted all cellular material soluble in sodium dodecylsulphate, and was applied directly to SDS-polyacrylamide gels, or frozen until required, whilst the pellet was discarded.

Polyacrylamide gel electrophoresis.

0.5 ml of *N. glutinosa* extract was applied to a 0.7 mm thick 16 x 14 cm discontinuous 10% polyacrylamide slab gel, in which the sample well consisted of a 12 cm continuous slot, with a single separate well at the end, which was used for molecular weight markers (Sigma, high molecular weight), which were dissolved in 2 ml of SDS-PAGE sample buffer.

Gels were modified after Laemmli (1970) and consisted of a separating gel of 9.67% acrylamide, 0.33% N,N'-methylenebisacrylamide, 0.1% sodium dodecylsulphate, 0.625% ammonium persulphate, 0.03% N,N,N',N'-tetramethylethylenediamine, 400 mM Tris, pH 8.8. The

stacking gel was of 4.35% acrylamide, 0.15% N,N'-methylene-bisacrylamide, 0.1% sodium dodecylsulphate, 0.12% ammonium persulphate, 0.06% N,N,N',N'-tetramethylethylenediamine, 125 mM Tris, pH 6.8. Gels were degassed for 15 min prior to polymerisation, which was initiated by addition of the N,N,N',N'-tetramethylethylenediamine. The electrode buffer was 192 mM glycine, 25 mM Tris, 0.1% sodium dodecylsulphate.

Gels were run at 25 mA for 30 min, until the bromophenol blue from the sample buffer had entered the gel, and subsequently at 50 mA/gel until the dye was within 1 cm of the bottom of the separating gel (ca. 2h). Alternatively, the gels were run overnight at 5 mA/gel.

#### Electroblotting.

Electroblotting of the proteins from the gel to nitrocellulose was performed at room temperature, using 80 mM Tris, 13 mM glycine, 20% (v/v) methanol transfer buffer, pH 9.2, in a Hoefer (San Francisco, California) Transphor cell. After running, individual slab gels were removed from their cassettes, and each placed in contact with a sheet of nitrocellulose (0.45  $\mu$ m pore size, Schleicher and Schuell, Keene, New Hampshire), which was prewetted with transfer buffer, and cut to the size of the gel. The gel and nitrocellulose were placed between double layers of filter paper in the transfer cassette, care being taken to avoid trapping air between any of the layers. The cassette was placed in the transfer unit and blotted for 3 h at 50-60 V constant voltage, with current rising from 300 to 500 mA.

After blotting, the part of the nitrocellulose bearing the molecular weight marker proteins was removed and stained for 15 min with 0.1% (w/v) amido black in 20% (v/v) methanol, 7.5% (v/v) acetic acid in water, and destained for 30 min in distilled water to reveal protein bands.

#### Immunodetection.

The rest of the nitrocellulose sheet was incubated for 30 min at room temperature with continuous agitation on a rocker platform in 5% (w/v) bovine serum albumin (BSA, Fraction V, Miles Scientific, Naperville, Illinois) in PBS-azide to block unfilled protein binding sites. The sheet was cut into 5 mm wide vertical strips, which were transferred to individual wells of a multiwell incubation tray (fraction collector rack tray, LKB, Bromma, Sweden). To each well was added 200  $\mu$ l of hybridoma supernatant in 4.5 ml of 1% BSA (w/v) in PBS-azide. Trays were covered and incubated overnight at room temperature with continuous agitation.

Each strip was washed for 30 min with four changes of 5 ml of 0.05% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20) in PBS-azide, after which it was incubated for 2 h with 4.5 ml of  $I^{125}$ -labelled rabbit anti-mouse antibody (prepared as in chapter 3) diluted 1:500 in PBS-azide containing 1% (w/v) BSA. The strips were washed with five changes over 1 h of 5 ml of Tween-PBS-azide, dried and exposed to Kodak (Rochester, New York) XRP-1 film at  $-70^{\circ}\text{C}$  for 5-20 h using an intensifying screen (Cronex, Du Pont, Willmington, Delaware). Film was developed using a Kodak X-Omat M20 processor.

#### In vivo labelling/immunoprecipitation.

Since UMI medium contains exogenous amino acids, N. glutinosa cells were grown in MS medium, supplemented with the vitamins and hormones of UMI medium. Four days after subculture, an aliquot of cells was removed sterilely from a flask, collected on a #60 sieve, and 2 g of cells weighed into a 25 ml conical flask, to which was added 5 ml of fresh MS medium. This flask was incubated for 12-18 h at  $27^{\circ}\text{C}$  in the dark at 125 rpm agitation, prior to addition of 1 mCi of L-[3,4,5- $H^3$ ]-leucine (New England Nuclear, Boston, Massachusetts) or 200  $\mu$ Ci of L-[ $S^{35}$ ]-methionine (Amersham, Arlington Heights, Illinois) in 200  $\mu$ l of MS medium. The cells were incubated for a further 18 h prior to harvesting on miracloth. The cells were washed briefly with

distilled water prior to grinding for 5 min at 4°C in a pestle and mortar in 1% (v/v) Nonidet P-40 (NP-40) in PBS (1 ml/g fresh weight of cells). The homogenate was allowed to stand 30 min at 4°C, then centrifuged at 100 000 g<sub>av</sub> for 1 h at 4°C to pellet undissolved material. The supernatant was used for immunoprecipitation.

50 ul of NP-40 extract was added to 100 ul of hybridoma supernatant, containing 23 ul of a 1:10 dilution of normal mouse serum in 1% (v/v) NP-40 in PBS. After standing for 30 min at room temperature, 100 ul of partially purified goat anti-mouse antibody (a gift from Dr. Ian Trowbridge) was added, and left to stand overnight at 4°C. Immune complexes were pelleted for 30 min at 1000 g<sub>max</sub> at 4°C and washed similarly three times with 0.4 ml of 0.2% (v/v) NP-40 in PBS. The final pellets were each dissolved in 20 ul of SDS-PAGE sample buffer, boiled and loaded into individual wells of a 1.5 mm thick 10% polyacrylamide gel, which was of composition and run under conditions as described above. One well in each gel contained C<sup>14</sup>-methylated molecular weight marker proteins (Amersham). After running, gels were fixed overnight in 50% (v/v) methanol, 5% (v/v) acetic acid in water, impregnated for 2 h with Enhance (New England Nuclear, Boston, Massachusetts), placed in water for 30 min to precipitate the fluor and dried under vacuum for 90 min at 60°C on a Hoefer gel drier. Gels were exposed to Kodak XAR-5 film for 20-40 h at -70°C, and the film developed as above.

#### Two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis was performed by the method of Garrels (1979). 140 mm long x 1 mm diameter isoelectric focussing rod gels were formed of 4% (v/v) NP-40, 9.15 M urea, 2% (v/v) pH 3.5-10.0 ampholines (LKB, Bromma, Sweden), and 4% acrylamide. Polymerisation was initiated by addition of ammonium persulphate to 0.083% (w/v). Gels were overlaid with 5 ul of 9.95 M urea, 4% (v/v) NP-40, 2% (v/v) ampholines, 100 mM dithiothreitol (2D sample buffer), and prefocussed for 15 min at 200 V, 15 min at 300 V and 30 min at 400 V. Immunoprecipitates from the above procedure were dissolved in 2D sample

buffer and a 5 ul aliquot applied to the top of the gel. The electrode buffers were 0.01 M  $\text{H}_3\text{PO}_4$  and 0.1 M NaOH. Electrophoresis was for a minimum of 1400 V-h. After the isoelectric focussing step, gels were equilibrated with 3% (w/v) sodium dodecylsulphate, 375 mM Tris, 50 mM dithiothreitol, pH 8.8, placed over 10% slab gels of composition as described above (without stacking gels), and sealed in place with 1% (w/v) agarose containing 0.005% (w/v) bromophenol blue. Electrophoresis and processing for fluorography were as described above.

## RESULTS.

### Western Blotting.

Of a total of 146 clones from the three hybridoma libraries raised in the laboratory against Nicotiana species (Chapter 3), which were analysed by Western blotting, 32 were found to recognise one or more protein bands on Western blots (Fig. 5.1). These could be grouped into two types according to their pattern of binding as assessed immunofluorescence microscopy: those which recognised epitopes exhibited on the exterior of the plasmalemma, and those showing no fluorescence on protoplasts or cells. The first group contains a number of clones raised against N. tabacum protoplasts which secrete antibodies showing both protoplast surface and cell wall fluorescence (Table 5.1).

In general, the plasmalemma positive clones detect polypeptides of high molecular weight, which run as disperse bands in polyacrylamide gels. In contrast, only three of 19 "internal" clones (Np 1.2D4, Np 3.1B4, Nt 4.1D7) recognise disperse bands. Two (Np 2.1D6, Np 3.4A6) recognise more than one polypeptide with substantial molecular weight differences. A number of clones (eg: Nt 3.3C2, Nt 3.5E7) recognise polypeptides which do not enter the separating gel at all, but remain in the stacking gel, or on the stacking-resolving gel interface.

There are a number of groups of clones showing similar patterns of recognition. Such is the case for clones Nt 3.2B4, Nt 4.2A4, Nt 4.3E5 and Nt 4.4E4, which also show similar patterns of immunofluorescence activity, binding both to intact cells and to protoplast plasmalemma. Another group of clones comprises Np B, 16.2C6, 16.4A6 and 16.4B4, whilst another includes 16.1B3 and 16.3C1. A number of clones recognising internal antigens also show similarities in pattern, eg: Np 3.1B2, Np 3.1B6 and Np 3.1C6; Nt 3.3C2 and Nt 3.5E7; Np 2.2C4 and Nt 4.1D1.

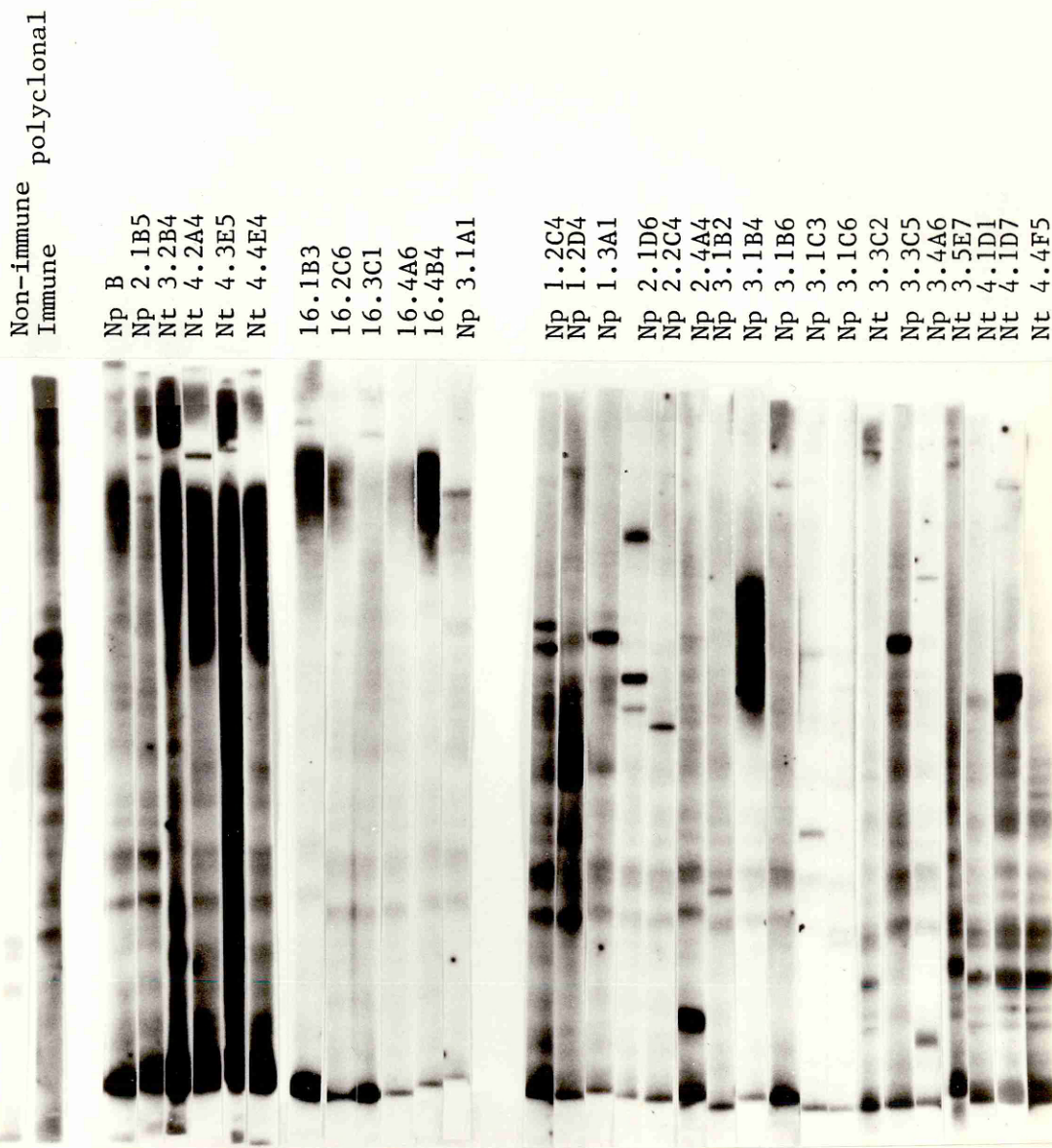


Figure 5.1. Western blot analysis of hybridoma supernatants.

Nitrocellulose strips were incubated with hybridoma supernatants as indicated. Detection of antibody binding was by  $I^{125}$ -labelled rabbit anti-mouse antibody binding and autoradiography as described. Molecular weight markers are not shown, since strips are cut from different gels.



Table 5.1. Western blot positive clones.

	CLONE	MOLECULAR WEIGHT OF ANTIGEN/kd			
Surface Immuno- fluorescence Positive	Np B	130-270			
	Np 2.1B5	175		*	**
	Nt 3.2B4	<15 48-62 82-200		*	** ***
	Nt 4.2A4	<15 48-62 82-200		*	** ***
	Nt 4.3E5	<15 48-62 82-200		*	** ***
	Nt 4.4E4	<15 48-62 82-200		*	** ***
	16.1B3	<15 130-230		*	**
	16.2C6	130-230			
	16.3C1	<15 130-230		*	**
	16.4A6	130-230			
	16.4B4	130-230			
	Np 3.1A1	195			
Surface Immuno- fluorescence Negative	Np 1.2C4	74			
	Np 1.2D4	45-54			
	Np 1.3A1	88			
	Np 2.1D6	60 70 170			
	Np 2.2C4	56			
	Np 2.4A4	23			
	Np 3.1B2	29			
	Np 3.1B4	61-130			
	Np 3.1B6	31			
	Np 3.1C3	38		****	
	Np 3.1C6	29.5			
	Nt 3.3C2			*	
	Np 3.3C5	65		****	
	Np 3.4A6	22 105			
	Nt 3.5E7			*	
	Nt 4.1D1	56			
	Nt 4.1D7	58-62		**	
	Nt 4.4F5	43			

Notes: \* Migrates into stacking gel only  
 \*\* Migrates to stacking-main gel interface  
 \*\*\* Also shows immunofluorescence on cell wall  
 \*\*\*\* Immunoprecipitation positive

## Immunoprecipitation analysis.

In vivo labelling/immunoprecipitation detected considerably fewer proteins than did Western blotting. Of 133 clones from all three libraries, which were tested, only nine precipitated proteins. The results are shown in Figure 5.2 and summarised in Table 5.2. Two of the clones (Np 3.1C3 and Np 3.3C5) precipitate proteins which are also detected by these antibodies in Western blots of total cellular protein, and have similar molecular weights in both analyses. There is also a set of five clones (17.3A6, Np L, Nt 4.2D8, Nt 4.3F7 and Nt 4.4C3) which recognise proteins of similar apparent molecular weight. To assess whether these were the same antigen, two-dimensional gel electrophoresis was performed on proteins immunoprecipitated by clones 17.3A6, Np L and Nt 4.2D8. Proteins from immunoprecipitates were first separated in pH 3.5-10.0 isoelectric focussing rod gels, then on 10% sodium dodecylsulphate slab gels. Each immunoprecipitate gave a similar pattern of ca. 7 spots on the resultant fluorograms (Fig. 5.3), which had similar pI's and molecular weights, indicating that all three clones recognise the same family of proteins.

Clone 17.4A1 recognised a polypeptide of 84 kd and also one of ca. 300 kd. It is possible that the latter represents a covalently cross-linked tetramer of the former, which is resistant to reduction by beta-mercaptoethanol. None of the immunoprecipitable antigens were exhibited in the cell wall or on the exterior of the plasmalemma, as assessed by immunofluorescence assay.

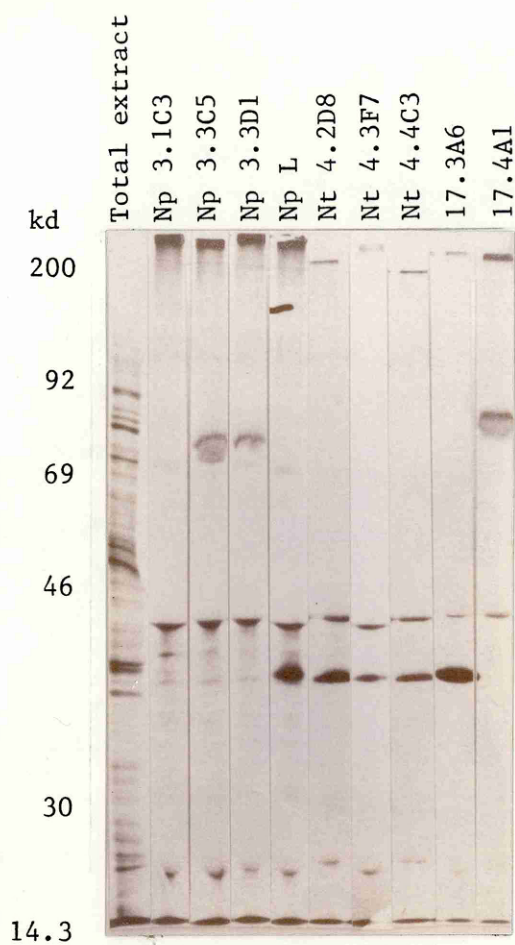


Figure 5.2. In vivo labelling/immunoprecipitation analysis of hybridoma supernatants.

Proteins were labelled by incubation of suspension culture cells with  $H^3$ -leucine for 18 h, prior to solubilisation with NP-40 and immunoprecipitation as described. Molecular weights are approximate, since proteins were run on different gels.

Table 5.2. In vivo labelling/immunoprecipitation positive clones.

CLONE	MOLECULAR WEIGHT OF ANTIGEN/kd	
Np 3.1C3	38	*
Np 3.3C5	72-76	*
Np 3.3D1	70-76	
Np L	36	
Nt 4.2D8	36	
Nt 4.3F7	36	
Nt 4.4C3	36	
17.3A6	36	
17.4A1	84 >200	

Notes: \* Also Western blot positive

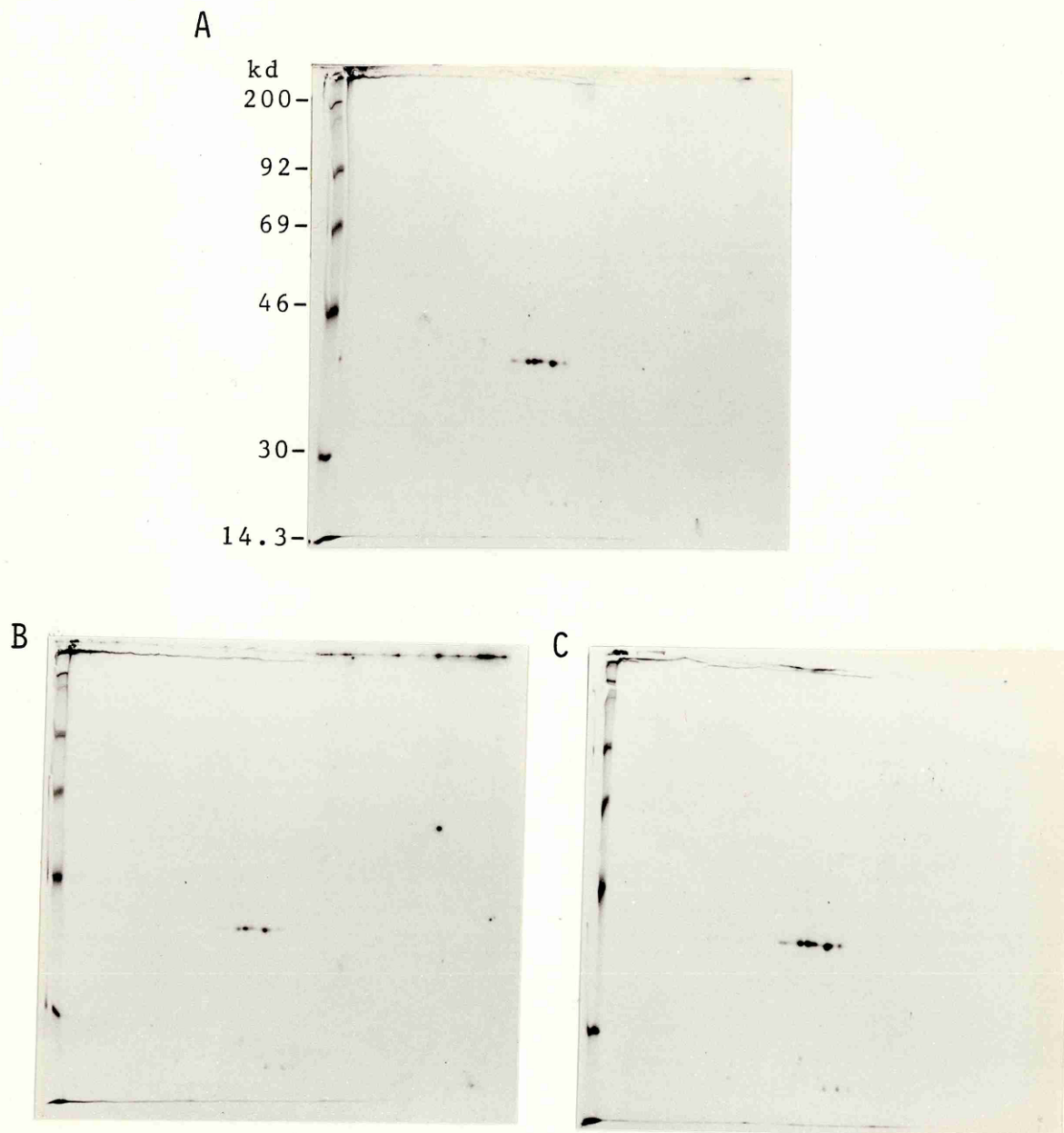


Figure 5.3. Two dimensional gel electrophoresis of in vivo labelled proteins precipitated by antibodies recognising a 36 kd protein.

Cellular proteins were labelled for 18 h with  $H^3$ -leucine, extracted with NP-40, and immunoprecipitated with (A) 17.3A6, (B) Np L, and (C) Nt 4.2D8. Immunoprecipitated proteins were resolved by two dimensional gel electrophoresis as described.

## DISCUSSION.

Western blotting and in vivo labelling/immunoprecipitation are two complementary procedures which allow detection of hybridomas which secrete antibody recognising epitopes on protein or glycoprotein antigens. Western blotting is expected to detect proteins which are in fairly high abundance, and for which the recognised epitopes are not labile to heat, to denaturation by sodium dodecylsulphate, or to reduction by beta-mercaptoethanol. Since total proteins are extracted from the cells, it is expected that detection will depend on the amount of protein present in the cells. Sodium dodecylsulphate is highly effective in solubilising proteins by binding to the polypeptide chain, stabilising it in a random coil conformation, and so might be expected to extract most cellular proteins (Gonenne and Ernst, 1978).

In contrast, in vivo labelling/immunoprecipitation requires that a protein be efficiently labelled with a specific amino acid for detection. Thus detection requires that the plant cells take up an exogenously supplied amino acid, that the protein has a significant rate of synthesis under the conditions of labelling, and contains residues of leucine or methionine. The detergent NP-40 is also less effective in solubilising membrane-bound proteins than is sodium dodecylsulphate (Gonenne and Ernst, 1978), so that less easily extractable (eg: more hydrophobic) proteins are unlikely to be detected. The major advantage of the method is that NP-40 will extract proteins at 4°C in essentially their native conformations. Thus any epitope that is labile to heat, sodium dodecylsulphate or reduction is more likely to be detected by this procedure than by Western blotting. That this is the case is demonstrated by clone 17.4A1, and the group of clones 17.3A6, Np L, Nt 4.2D8, Nt 4.3F7 and Nt 4.4C3, which all recognise protein epitopes resistant to this procedure, but not to Western blotting. It is probable that the epitopes recognised by these clones are labile to heat or one of the above reagents, and will not renature after the removal of these reagents whilst incubating the nitrocellulose filter with antibody in the immunodetection steps of

Western blotting.

72% of clones tested show no recognition properties for proteins either by Western blotting or by in vivo labelling/immunoprecipitation despite giving positive signals in the radioimmunoassay. There are two possible reasons for this. First, the antigens recognised might not be proteins. The plasmalemma also contains lipid and glycolipid components which are potentially antigenic, and would not be resolved by this method (Kannagi et al., 1982; Symington et al., 1984; Cheresch et al., 1984). In addition, plant cells elaborate complex carbohydrates, which would not be detected since they would neither be labelled by radioactive amino acids nor resolved by SDS-PAGE. The second potential cause of non-detection of an antigen is a deficiency in both detection procedures. If the recognised epitope is irreversibly denatured in the Western blotting procedure, and the protein bearing it is synthesised at a low rate, or the labelled amino acid is poorly represented in the primary structure, then it will escape detection by both techniques.

The detection of diffuse bands in Western blots by certain antibodies suggests that the proteins recognised are glycosylated (see chapter 6). Such a pattern might be due to microheterogeneity in extent of glycosylation, which would result in heterogeneity in molecular weight and also charge properties of the antigen. This is a common property of glycoproteins (Sharon and Lis, 1979). Modification of saccharide moieties, for example by sulphation (Lindahl et al., 1986), might also introduce charge heterogeneity, although sulphation of plant glycoproteins has not been demonstrated. The failure of some antigens even to enter the separating gel might represent an extreme form of these processes.

It is interesting that a number of proteins are recognised by antibodies secreted by more than one clone. It is expected that in a given immunogen preparation, a few epitopes will be immunodominant and elicit more B cell recognition than others (see Chapter 9). Six groups of clones may be recognised by Western blotting and immuno-

precipitation, representatives of each of which are Nt 3.2B4, 16.4B4, 16.1B3, Np 3.1B2, Np 2.2C4 and 17.3A6. Of these, the last two are most interesting, as clones have been raised against these antigens using different plant material, different immunisation schedules and different litters of mice, by different investigators. From these results and those recorded in Table 3.4, it is obvious that there is a high degree of inter-species homology of proteins within the genus Nicotiana. Further, 17.3A6 has been shown to recognise a protein of similar molecular weight which can be radiolabelled in Glycine max suspension cultures (not shown).

Thus, by using appropriate procedures, a number of different protein antigens were detected. The hybridoma libraries show considerable heterogeneity of reactivity, but interestingly, rather limited specificity against plasmalemma proteins, which fall into six classes, and all show molecular weight heterogeneity. In the next chapter, further characterisation of the 16.4B4 antigen is described, which indicates the nature of the recognised epitope, and the structural characteristics of plasmalemma expressed proteins in plants.



CHAPTER SIX.

PURIFICATION AND FURTHER CHARACTERISATION OF A PLASMALEMMA ASSOCIATED  
ANTIGEN.

## INTRODUCTION.

A number of antibodies recognise determinants on molecules which will migrate into sodium dodecylsulphate polyacrylamide gels, suggesting that they are proteinaceous in nature. This renders them amenable to further study by well established techniques. Of particular interest are those antigens which are expressed on the plasmalemma. Amongst antibodies raised to Nicotiana glutinosa, two plasmalemma specificities are seen, exemplified by monoclonal antibodies 16.1B3 and 16.4B4. The antigen recognised by the latter is seen as a heterogeneous species of 130-230 kd molecular weight in polyacrylamide gels. It was suggested in Chapter 5 that this was indicative of heterogeneous glycosylation. In this chapter, some of the properties of the 16.4B4 antigen are further investigated, resulting in a greater characterisation of the nature of this antigen.

In parallel to the studies of the 16.4B4 antigen, some of the experiments were performed using the antigen recognised by monoclonal antibody 17.3A6. This is highly labelled in vivo by growth of suspension cultures in MS medium containing radioactive methionine or leucine, and is readily immunoprecipitable. It is not detected in immunoblots, however, indicating that the epitope recognised by 17.3A6 is labile to heat and/or denaturing detergents. Thus the strategies used for purification of the 16.4B4 and 17.3A6 antigens differ in some respects to compensate for the different recognition properties of the antibodies, and the different physical properties of the antigens. In this chapter, both 16.4B4 and 17.3A6 antigens are purified and their properties further investigated.

## MATERIALS AND METHODS.

### Immunodetection.

Immunodetection methods were adapted to use horseradish peroxidase conjugated goat anti-mouse antibody rather than  $I^{125}$ -labelled rabbit anti-mouse antibody. Antibody reactivity was detected by chromogenic oxidation of 4-chloro-1-naphthol, which was deposited as a purple precipitate on the filter. This method was found to be as sensitive as use of freshly iodinated antibody, without the problems inherent in handling radioactive material and caused by decay of the radioactivity and denaturation of the iodinated antibody (see Chapter 3).

Following transfer to nitrocellulose, excess protein binding sites were blocked by incubation for 30-60 min in 5% (w/v) BSA in Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris, pH 7.5). Incubation with antibodies was at 1:25 dilution for culture supernatants, 1:500 for 16.4B4 purified from ascites, and 1:250 for polyclonal antisera. All dilutions were into 1% (w/v) BSA in TBS. Following incubation with antibody overnight at room temperature with agitation on a rocker platform (Bellco, Vineland, New Jersey), filters were washed with three changes of 5 min each of 0.05% (v/v) Tween 20 in TBS. Filters were then incubated for 2 h in peroxidase conjugated goat anti-mouse antibody (BioRad, Richmond, California) diluted 1:3000 in TBS containing 1% (w/v) BSA. They were next washed in three 5 min changes of 0.05% (v/v) Tween 20 in TBS, then in 3-5 changes of TBS over 5 min. Antibody binding was visualised by incubating the filters for up to 30 min in a solution of 0.5 mg/ml 4-chloro-1-naphthol and 0.015% (v/v)  $H_2O_2$  in 17% (v/v) methanol in TBS. The reaction was stopped by washing 2 or 3 times in distilled water. Filters were photographed using Kodak (Rochester, New York) Plus-X pan 125 film, with overhead illumination.

#### Periodate oxidation of antigens.

After polyacrylamide gel electrophoresis, material was immobilised on nitrocellulose filters as described in Chapter 5. Periodate oxidation of sugar residues of glycoproteins bound to the filters was performed as described by Woodward et al. (1985). After blotting, filters were washed twice with 50 mM sodium acetate, pH 4.5, after which test strips were incubated for 1 h in the dark in a fresh solution of sodium periodate (usually 10-20 mM) in the same buffer. Filters were washed twice in sodium acetate buffer and twice in TBS. Aldehyde groups generated by oxidation were blocked by incubation for 30 min in 1% (w/v) glycine in TBS, to form Schiff bases. Blots were washed twice more in TBS, before immunodetection as described.

#### Silver staining of proteins in polyacrylamide gels.

Silver staining was performed by the method of Merrill et al. (1981) as modified by Morrissey (1981). After electrophoresis, gels were fixed for at least 30 min in 40% (v/v) methanol, 10% (v/v) acetic acid in water. Secondary fixation was for at least 30 min in two changes of 10% (v/v) ethanol, 5% (v/v) acetic acid in water, following which gels were incubated for 30 min in 10% (v/v) glutaraldehyde in water and rinsed overnight in several changes of distilled water. They were next incubated for 30 min in 5 mg/l dithiothreitol, followed by 20 min in 2 g/l silver nitrate. After brief washing in water, protein bands were developed to the appropriate intensity in 3% (w/v)  $\text{Na}_2\text{CO}_3$ , 0.1% (v/v) formaldehyde in water. Development was stopped in 5% (v/v) acetic acid, and gels photographed on a light box using Kodak Plus-X pan 125 film.

#### Detergent solubility and protease sensitivity.

A number of detergents were tested for their ability to solubilise the 16.4B4 and 17.3A6 antigens from cells of N. glutinosa. Cells from 5 day old suspension cultures were harvested and washed as described, and 1 g amounts extracted with 2 ml of solutions of various

detergents, using a pestle and mortar. Conditions were Tris buffered saline, 1% (v/v) NP-40 in TBS and 1% (w/v) CHAPS (BioRad, Richmond, California) in TBS, all at 4°C for 1 h, followed by centrifugation at 100 000 g<sub>av</sub> for 1 h at 4°C to remove insoluble debris. Extraction with 1% (w/v) SDS at room temperature for 5 min was followed by heating the homogenate to 100°C for 10 min, and centrifugation as above at 25°C. An aliquot of each detergent extract was diluted 1:1 with SDS-PAGE sample buffer, boiled and resolved on a 10% polyacrylamide gel, prior to electroblotting and probing with purified 16.4B4 monoclonal antibody (prepared as in Chapter 3), and 17.3A6 polyclonal antiserum (see below).

Samples were run under non-reducing conditions by denaturation in SDS-PAGE sample buffer from which beta-mercaptoethanol was omitted. Proteinase K treatment was performed in SDS-PAGE sample buffer prior to running the gel, by incubation at 60°C for 1 h with one tenth volume of 10 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, Indiana) in SDS-PAGE sample buffer. The sample was subsequently heated to 100°C for 3 min prior to loading onto the gel and electrophoresis.

16.4B4 immunoaffinity chromatography.

16.4B4 antibody purified from ascites fluid as described in Chapter 3 was passed over a 10 ml BioGel P6 DG column (BioRad, Richmond, California) to exchange the buffer for 0.5 M NaCl, 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 8.3. The antibody peak was detected by measurement of OD<sub>280</sub> of the eluent. Antibody was diluted in this buffer to 5 mg/ml.

Cyanogen bromide activated CH-sepharose 4B (Pharmacia, Uppsala, Sweden) was swollen for 15 min in 1 mM HCl as described by the manufacturer (Pharmacia, 1979). Antibody solution at 5 mg/ml was coupled with swollen gel, by end over end rotation at room temperature for 2 h. Excess coupling sites in the gel were blocked by incubation with 0.2 M glycine, pH 8.0 for 2 h at room temperature. The gel was then washed three times alternately with 0.5 M NaCl, 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 8.3 and 0.5 M NaCl, 0.1 M sodium acetate, pH 4.0 to elute

non-specifically bound protein. A column was made of 1-2 ml of antibody-sepharose and equilibrated with 1% (v/v) Triton X-100, 0.2% (w/v) SDS in PBS.

Detergent soluble material was extracted from plant cells, and subjected to immunoaffinity chromatography by a modification of the procedure described by Lazarides et al. (1984). 5 g of plant cells were harvested and ground at room temperature for 5 min in 1 ml/g fresh weight of 1% (w/v) SDS, 10 mM Tris, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, pH 8.0. The homogenate was boiled for 10 min, and insoluble material precipitated by centrifugation at 100 000  $g_{av}$  for 1 h at 25°C. The supernatant was diluted 1:10 with 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 130 mM NaCl, 20 mM Tris, 5 mM EDTA, pH 8.0. All subsequent steps were performed at 4°C. 40 ml of extract was passed down the antibody-sepharose column, which was washed with 10-20 column volumes of 1% (v/v) Triton X-100, 0.2% (w/v) SDS in PBS, before elution of specifically bound material with 1% (v/v) Triton X-100, 0.2% (w/v) SDS in 50 mM glycine, pH 2.5. 0.5 ml fractions of eluted material were collected; 20  $\mu$ l aliquots of each fraction were diluted 1:1 with SDS-PAGE sample buffer. These were subjected to electrophoresis, followed by silver staining or electroblotting; blots were probed with purified 16.4B4 antibody as described.

#### 17.3A6 immunoaffinity chromatography.

Antibody 17.3A6 purified from ascites fluid was coupled to cyanogen bromide activated Sepharose 4B as for 16.4B4. 5 g of N. glutinosa cells were extracted for 1 h at 0°C with 2 ml per 1 g fresh weight of 1% (v/v) NP-40 in PBS, prior to centrifugation at 100 000  $g_{av}$  for 1 h at 4°C. The supernatant was passed directly over the column, which was washed with 0.2% (v/v) NP-40 in PBS. Elution of absorbed material was with 0.2% (v/v) NP-40 in 50 mM glycine, pH 2.5. Aliquots of fractions from the eluted material were electrophoresed and silver stained as described above.

## Antibody production.

Immunoaffinity column eluent fractions which contained the 16.4B4 and 17.3A6 antigens, as determined by silver staining and immunoblotting of electrophoresed proteins were pooled and dialysed for 24 h against PBS. Dialysed material was diluted to 6 ml with PBS, and used to immunise mice. For each of the antigens, three female Balb/c mice were given four consecutive weekly injections of 0.5 ml of the diluted material. Three days after the final injection, mice were tail bled, and the serum clarified by centrifugation at 15 000  $g_{av}$  at 4°C for 15 min, prior to storage at -20°C. Mice immunised with the 16.4B4 antigen were subsequently sacrificed, and hybridomas generated as described in Chapter 3, except that after fusion cells derived from one mouse were plated into 384 0.5 ml cultures in HAT medium. These were fed after 3 days with a further 0.5 ml of HAT medium, and weekly thereafter by withdrawal of 0.5 ml of culture medium, and its replacement with a similar volume of fresh HAT medium. Expansion of actively growing cultures and freezing in liquid nitrogen were as described (Chapter 3). Cloning by limiting dilution was not performed.

Supernatants from frozen lines were assayed for secretion of antibody against *N. glutinosa* cell extracts by Western blotting of SDS solubilised material as described in Chapter 5, except that the enzyme linked second antibody method described above was used to detect antibody binding.

## Deglycosylation.

For chemical deglycosylation of the 16.4B4 antigen, 50 g of *N. glutinosa* cells were extracted into 50 ml of buffer as described for 16.4B4 immunoaffinity chromatography. The extract was applied without dilution to a 1000 mm long by 25 mm diameter column of Sephadex G-150, which had previously been equilibrated with 1% (w/v) SDS in TBS. The column was eluted with the same buffer, and 5 ml fractions collected. An aliquot of every third fraction was diluted 1:20 with TBS, and antigenic material detected on a slot blot using purified 16.4B4

antibody diluted 1:500 in 1% (w/v) BSA in TBS and peroxidase conjugated goat anti-mouse antibody as described above. Aliquots from the same fractions were diluted 1:1 with sample buffer without beta-mercaptoethanol and protein content resolved on polyacrylamide gels by silver staining. Fractions showing peak antibody binding were pooled and dialysed extensively against distilled water at room temperature. Residual SDS was removed by extraction of the dialysed sample with 5 volumes of isopentyl alcohol as described by Salcedo et al. (1983), and the aqueous phase lyophilised.

Chemical deglycosylation was performed as described by Edge et al. (1981). 1 ml of anisole and 0.5 ml of trifluoromethane sulphonic acid (both from Aldrich, Milwaukee, Wisconsin) were mixed at 0°C, and added to 10 mg of lyophilised protein. Argon was bubbled through the solution for 1 min, and the reaction allowed to continue at room temperature with mixing for 1 h, during which the solution turned deep red. The reaction was terminated by addition of a twofold excess of diethyl ether which had been cooled to -40°C, followed by an equal volume of 50% aqueous pyridine at 0°C. The solution was mixed by vortexing, and the ether phase removed. Ether extraction was repeated, and the aqueous phase dialysed against 4 l of 2 mM pyridine-acetate buffer, pH 5.5 for 24 h. The dialysed phase was lyophilised, and 2 mg of lyophilisate were dissolved in 1 ml of sample buffer for polyacrylamide gel electrophoresis.

#### Amino acid analysis.

For amino acid compositional analysis of the 16.4B4 antigen, after affinity chromatography as described above, further purification was effected at room temperature by passage of pooled peak fractions over a 300 mm long 20 mm diameter Sephadex G-150 (Pharmacia, Uppsala, Sweden) gel filtration column, as described for chemical deglycosylation. 2 ml fractions were collected and antigenic material was detected on a slot blot as described above. Antigen containing fractions were pooled, dialysed and residual SDS removed as described above. Amino acid analysis was performed by D.C. Karr (Peptide Biology



Laboratory, Salk Institute). The aqueous phase was lyophilised and the residue dissolved in 200  $\mu$ l of glacial acetic acid in a small vial. The acetic acid was removed by evaporation, and replaced by 25  $\mu$ l of 4 M methane sulphonic acid, 0.2% (w/v) tryptamine, containing 1 nmol of norleucine as internal standard (all from Aldrich). The vial was sealed and incubated at 110°C for 24 h to hydrolyse the protein. The hydrolysate was neutralised with 25  $\mu$ l of 3.5 M NaOH, and amino acid composition was determined using a Beckman 121 MB automated amino acid analyser equipped with a model 126 data system. Amino acids were eluted using the sodium citrate standard program (Beckman), and detected as their ninhydrin adducts (Spiess et al., 1982).

#### Time course of antigen accumulation.

The time courses of accumulation of the 16.4B4 and 17.3A6 antigens were determined by extraction into sample buffer of aliquots of cells at different times during the growth of the culture, followed by resolution on polyacrylamide gels and Western blotting. On each day after subculture for 14 days, cells were harvested on miracloth and frozen in liquid nitrogen as described (Chapter 2). A 1 g aliquot from each time point was homogenised with a pestle and mortar for 1 min at room temperature in 2 ml of SDS-PAGE sample buffer, before boiling for 5 min. Insoluble debris was pelleted by centrifugation at 100 000  $g_{av}$  for 1 h at 25°C, following which a 20  $\mu$ l aliquot of the supernatant from each fraction was subjected to polyacrylamide gel electrophoresis and Western blotting as described (Chapter 5). Filters with immobilised proteins were incubated with purified 16.4B4 antibody at 1:250 dilution in TBS-1% BSA, or polyclonal 17.3A6 antiserum at 1:2000 dilution in the same solution, followed in both cases by detection using peroxidase conjugated goat anti-mouse antibody as described above.

## RESULTS.

### Affinity chromatography.

Fig. 6.1A shows a silver stained gel of fractions eluted from the 16.4B4 immunoaffinity column, and Fig. 6.1B a Western blot of the same fractions probed with purified 16.4B4 antibody. A peak of elution of the antigen is seen in the Western blot after adjusting the eluent pH to 2.5. This occurs in the fraction showing the maximum degree of diffuse silver staining in Fig. 6.1A. The band silver stains poorly, but contains a single, sharper band which stains more strongly. There is little silver staining elsewhere in the gel, with the exception of two diffuse bands which are seen in all lanes, which are an artifact due to the presence of beta-mercaptoethanol, which was included in the SDS-PAGE sample buffer to reduce the proteins in the sample prior to running the gel. Such bands are not seen when dithiothreitol is used as reductant. The lack of other silver staining indicates that the protein is eluted in essentially pure form.

A similar conclusion is reached for affinity chromatography on 17.3A6-sepharose (Fig. 6.2). Apart from a faint band of 24 kd, no other eluted material is seen. The 17.3A6 antigen is silver stained far more strongly than the 16.4B4 antigen, and runs as a discrete, sharp band, as opposed to the diffuse band of the latter. Since no reactivity with 17.3A6 monoclonal antibody was seen on Western blots (Chapter 5 and below), the eluted material was not probed for antibody reactivity.

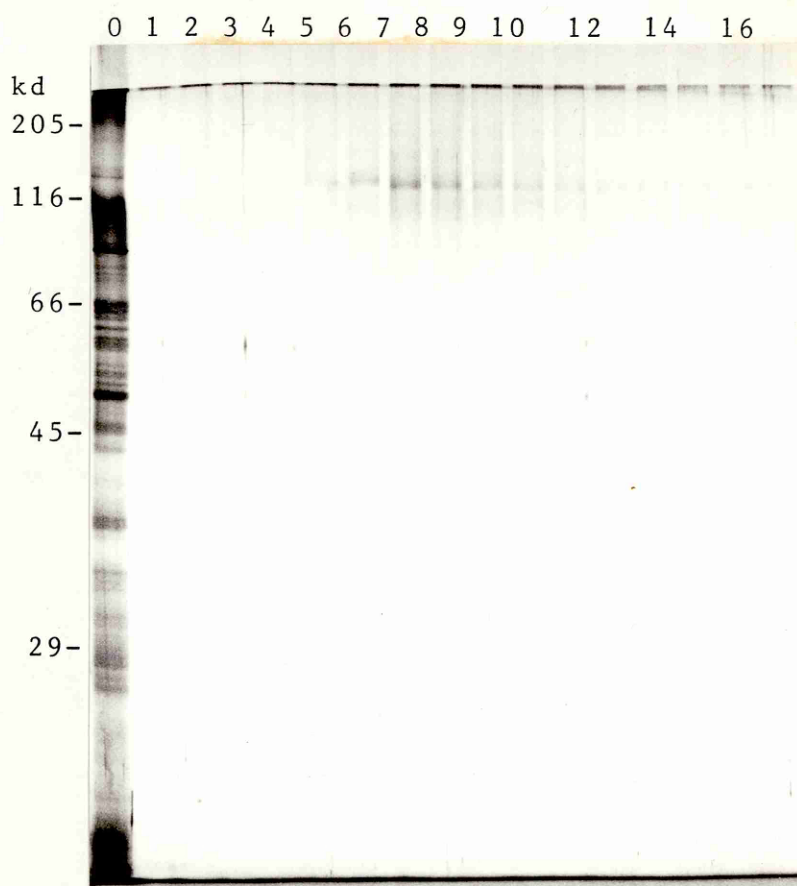


Figure 6.1A. Silver stained gel of fractions eluted from a 16.4B4 immunoaffinity column.

Aliquots of fractions eluted from the immunoaffinity column upon application of pH 2.5 buffer were subjected to silver staining. Lane 0: total SDS extract of *N. glutinosa* cells. Lanes 1-16: consecutive 0.5 ml fractions of column eluent after application of pH 2.5 buffer.

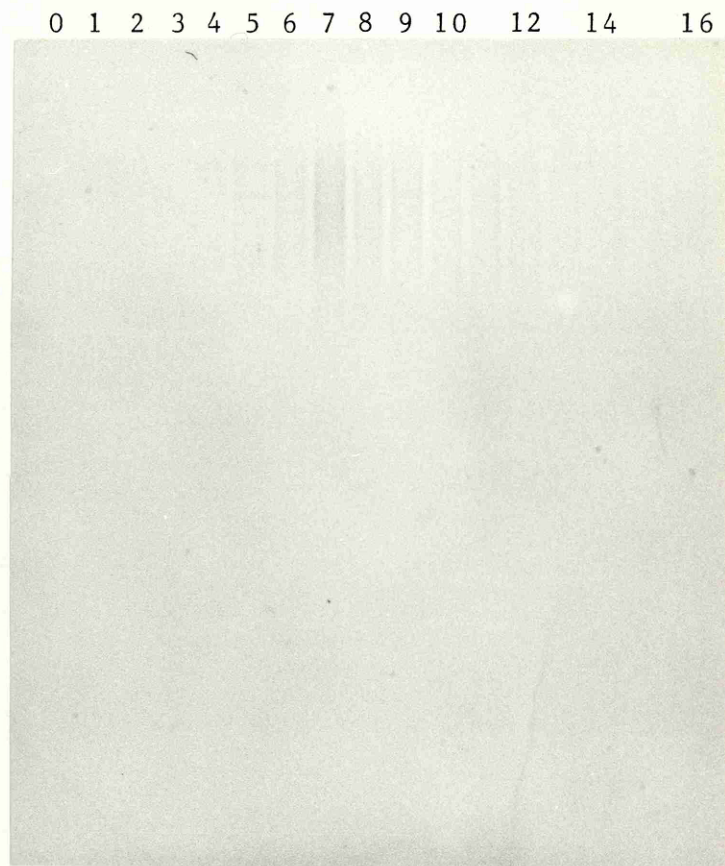


Figure 6.1B. Western blot of proteins eluted from a 16.4B4 immunoaffinity column.

Aliquots of fractions eluted from the immunoaffinity column upon application of pH 2.5 buffer were resolved by SDS-PAGE, immobilised on a nitrocellulose filter and probed with purified 16.4B4 antibody. Lane 0: total SDS extract of *N. glutinosa* cells. Lanes 1-16: consecutive 0.5 ml fractions of column eluent after application of pH 2.5 buffer.

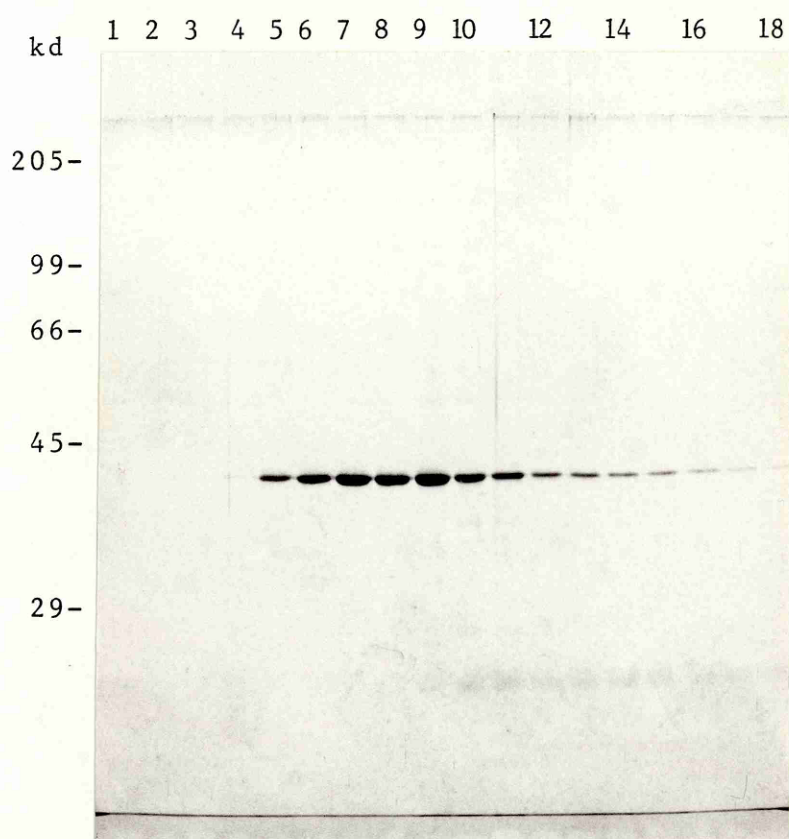


Figure 6.2. Silver stained gel of fractions eluted from a 17.3A6 immunoaffinity column.

Lanes 1-18: successive 0.5 ml fractions of eluent after application to the column of pH 2.5 buffer.

## Polyclonal and monoclonal antibodies.

Fig. 6.3 is a Western blot showing the reactivity of monoclonal antibody 16.4B4 and of polyclonal antisera resulting from immunisation of three mice with purified 16.4B4 antigen and the residual reactivity after periodate treatment of the antigenic material. Whereas the monoclonal antibody recognises a single diffuse band of 130-230 kd molecular weight on Western blots, the pattern of reactivity of polyclonal antisera is considerably more complex. All three antisera show the high molecular weight disperse band characteristic of monoclonal antibody 16.4B4, as well as a lower molecular weight diffuse band of 50-92 kd, and a series of sharper bands superimposed on these. All three polyclonal antisera bind to a sharper band of ca. 50 kd, whilst sera 25 and 26 both show sharper bands of ca. 40 kd. Serum 26 also shows a discrete band of 87 kd, and there is some reactivity in the stacking gel for all antisera.

Fig. 6.3 also shows the result of periodate treatment of the blots prior to probing with 16.4B4 monoclonal antibody or with polyclonal antisera. Administration of 10 mM or 50 mM periodate completely abolishes binding of 16.4B4 monoclonal antibody to blots. Merely incubating the filter in 50 mM sodium acetate buffer, pH 4.5, does not have this effect, which it is concluded is due to periodate oxidation of the epitope recognised by monoclonal antibody 16.4B4. In contrast, binding of polyclonal antisera to the series of recognised antigens is attenuated but not abolished by this treatment. In particular, the sharper bands seen in the pattern show slight periodate sensitivity, whilst the 130-230 kd band was substantially reduced by periodate treatment. The 50-92 kd diffuse band was intermediate in periodate sensitivity.

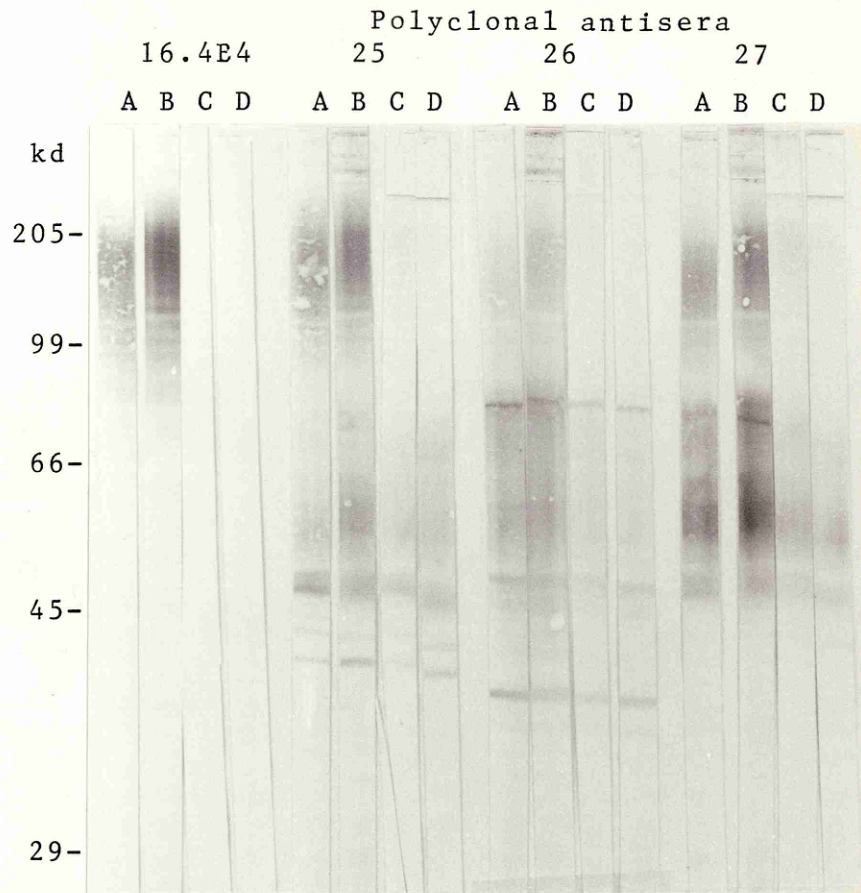


Figure 6.3. Immunoreactivity and periodate sensitivity of antigens recognised by 16.4B4 monoclonal antibody and anti-16.4B4 antigen polyclonal antisera.

Western blot of SDS extract of *N. glutinosa* cells probed with 16.4B4 monoclonal antibody and polyclonal antisera 25, 26 and 27 raised against purified 16.4B4 antigen. Filters were treated before antibody incubations as below: A) controls at pH 7.2; B) washed 1.5 h in pH 4.5 buffer; C) 10 mM periodate treated, 1 h; D) 50 mM periodate treated 1 h.

In contrast to the results for 16.4B4, immunisation with purified 17.3A6 antigen results in production of antisera reactive with essentially one band in the blot (Fig. 6.4). This has the same molecular weight (36 kd) as the 17.3A6 antigen detected by immunoprecipitation analysis (Chapter 5). Serum 30, which shows the most pronounced staining of the antigen, also shows a series of fainter bands of lower molecular weight than the major species. No reactivity of the monoclonal antibody was seen in this assay. Binding of polyclonal antisera to the 17.3A6 antigen was insensitive to periodate (result not shown).



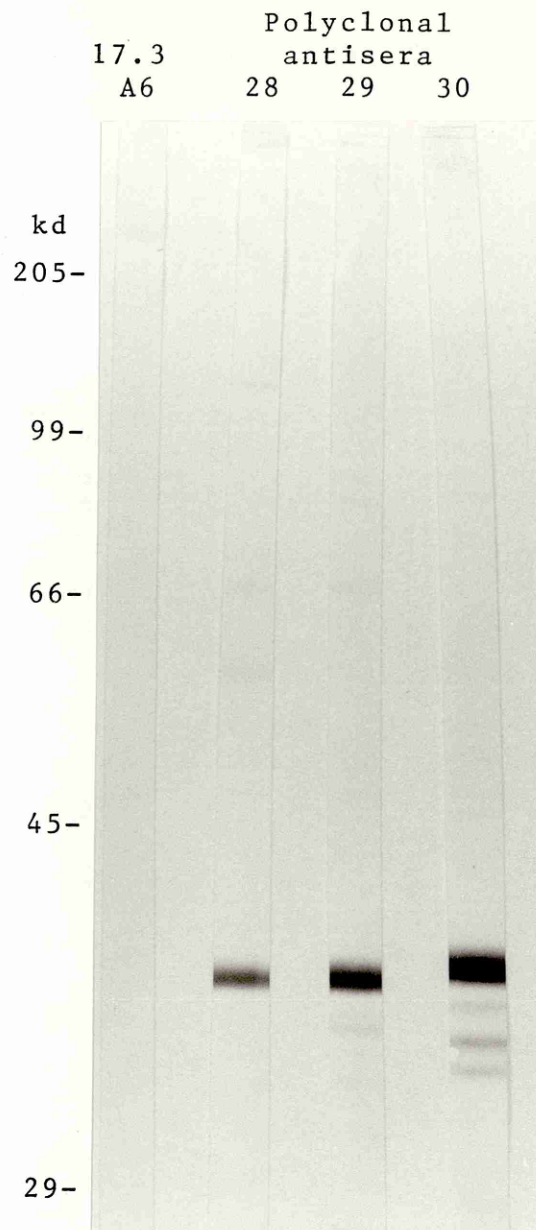


Figure 6.4. Immunoreactivity of 17.3A6 monoclonal antibody and anti-17.3A6 antigen polyclonal antisera.

SDS extract of *N. glutinosa* cells resolved by SDS-PAGE was transferred to nitrocellulose and probed with 17.3A6 monoclonal antibody and polyclonal antisera 28, 29 and 30 raised against purified 17.3A6 antigen.

The fusions using mice immunised with the 16.4B4 antigen produced 103 hybrids which grew in HAT medium, and reached sufficient density on expansion to be frozen. Of these, 15 showed binding on Western blots to material from *N. glutinosa*. Figure 6.5 shows a representative sample of the reactivities generated. These may be grouped into three classes. The first binds to high molecular weight diffuse bands, and is exemplified by 27.1E3 which shows a pattern similar to 16.4B4. 26.2E5 shows this reactivity and also reactivity to a lower molecular weight diffuse band and to material which runs into the stacking gel only. Antibodies 25.6D4 and 27.5A2 bind to a large number of more discrete lower molecular weight bands in the gel. In contrast, a number of antibodies (eg: 25.1C2) bind to essentially a single band in the gel, which gives a weak signal in all cases. Most of this last class bind to a band of 50 kd molecular weight, but 27.1D7 also binds to a band of lower molecular weight, whilst 27.8F2 binds principally to a band of 41 kd.

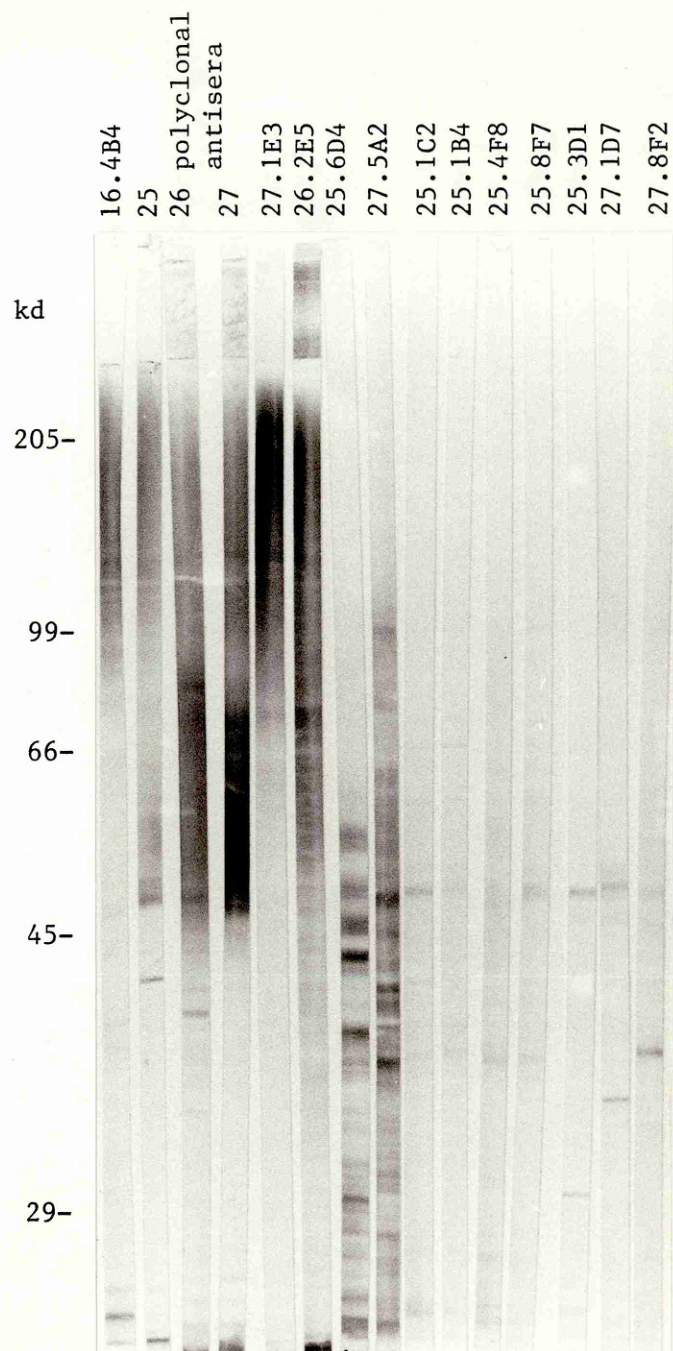


Figure 6.5. Reactivity of monoclonal antibodies raised against purified 16.4B4 antigen.

Strips from filters with transferred proteins were probed with monoclonal antibodies as indicated.

Other treatments of Western blots.

Figure 6.6A shows the detergent extractability of the 16.4B4 antigen. Boiling in sodium dodecylsulphate was found to be the most effective method of solubilisation of the antigen. Of the other detergents tried, CHAPS at 4°C was the most effective, but substantial antigenic material was also extracted by NP-40 at this temperature. A small quantity of the antigen was extracted by grinding the cells in buffer alone at 4°C. Extraction with NP-40 or CHAPS at 4°C resulted in a different pattern of reactivity on Western blots to that seen upon boiling in SDS, with a concentration of the reactivity in a more discrete band of high molecular weight, and less reactivity to the lower molecular weight heterogeneous portion.

In contrast, the 17.3A6 antigen (Fig 6.6B) was extracted quite effectively by buffer at 4°C, and as effectively by NP-40 or CHAPS at 4°C as by boiling in SDS. The pattern of polypeptides recognised on the blot was identical for the 17.3A6 antigen extracted by any of these methods.

Figure 6.6C shows the result of non-reduction of the sample before running the gel, and of proteinase K treatment. Whereas the former has no effect on the apparent molecular weight of the protein, proteinase K treatment prior to running the gel results in complete abolition of 16.4B4 antibody binding.

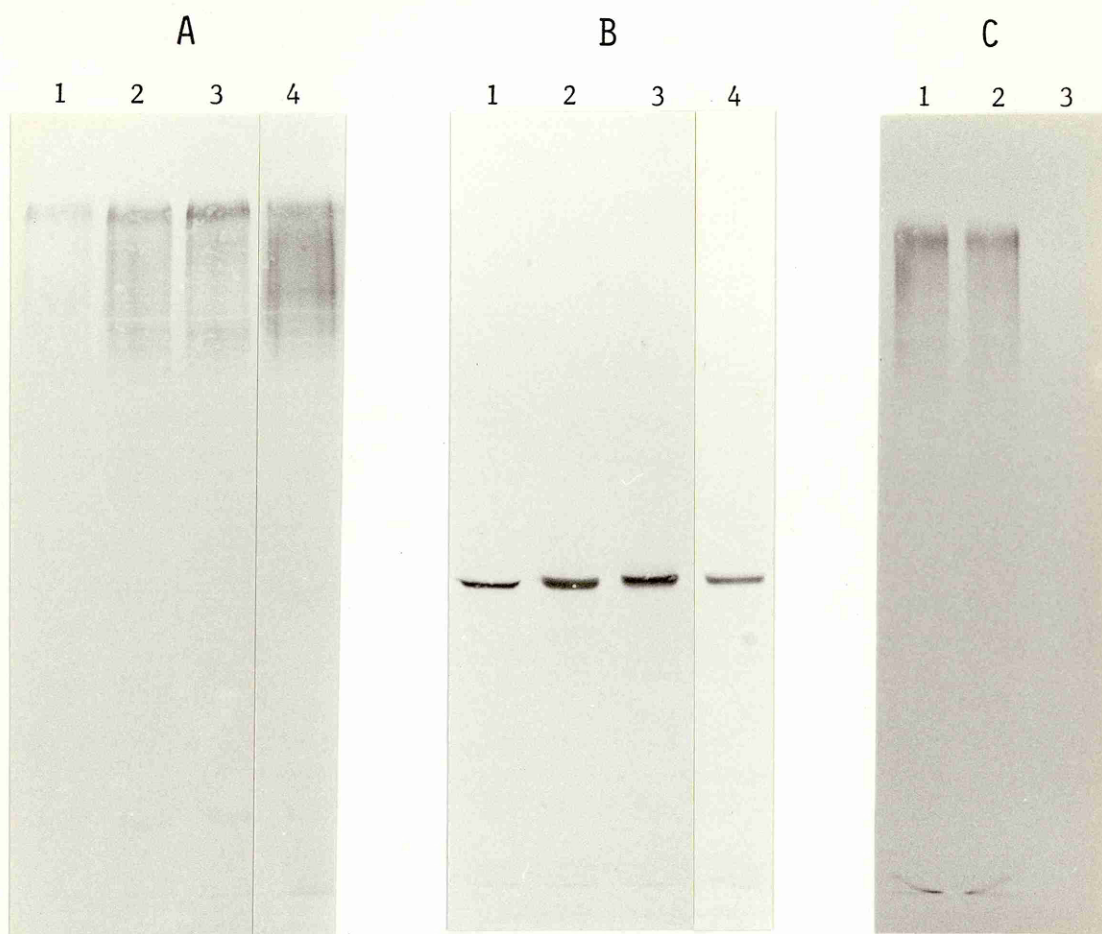


Figure 6.6. Detergent extractability of the 16.4B4 and 17.3A6 antigens, and reduction and protease sensitivity of the 16.4B4 antigen.

A and B: Immunodetection of antigen solubilised by: 1) TBS, 4°C; 2) 1% (v/v) NP-40, 4°C; 3) 1% (w/v) CHAPS, 4°C; 4) 1% (w/v) SDS, 100°C. A 20 µl aliquot from 1 g of *N. glutinosa* cells homogenised in 2 ml of detergent solution was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with (A) 16.4B4 monoclonal antibody or (B) 17.3A6 polyclonal antiserum.

C: Aliquots of protein extract were treated with: 1) complete SDS-PAGE sample buffer; 2) SDS-PAGE sample buffer without beta-mercaptoethanol; 3) proteinase K in sample buffer prior to loading gel.

## Chemical deglycosylation.

For efficient recovery of protein following chemical deglycosylation, it is necessary to use 5-10 mg of starting material, an amount which is not easily obtained by immunoaffinity chromatography (see below). Thus it was decided to use gel filtration on Sephadex G-150 to size fractionate the protein prior to deglycosylation, resulting in partial enrichment in the 16.4B4 antigen. Figure 6.7A lane 1 indicates that this strategy was only partially successful, apparently due to limited resolution achieved on the column. This may be due to some compression of the gel matrix as a result of employing such a large bed volume. Unfortunately, an uncharacterised component of the column chromatography conditions resulted in extensive smearing of the silver stained gels used to visualise the proteins, so that the extent of contamination with lower molecular weight proteins cannot be easily assessed. Discrete bands are seen in the gel, however, down to ca. 40 kd molecular weight. Disperse high molecular weight staining characteristic of the 16.4B4 antigen can also be seen.

In contrast, following deglycosylation the smearing evident in the non-deglycosylated sample is much reduced. A series of faint bands is seen, but the major feature of the silver stained gel is a discrete band of 50 kd molecular weight, which has no counterpart in the non-deglycosylated sample.



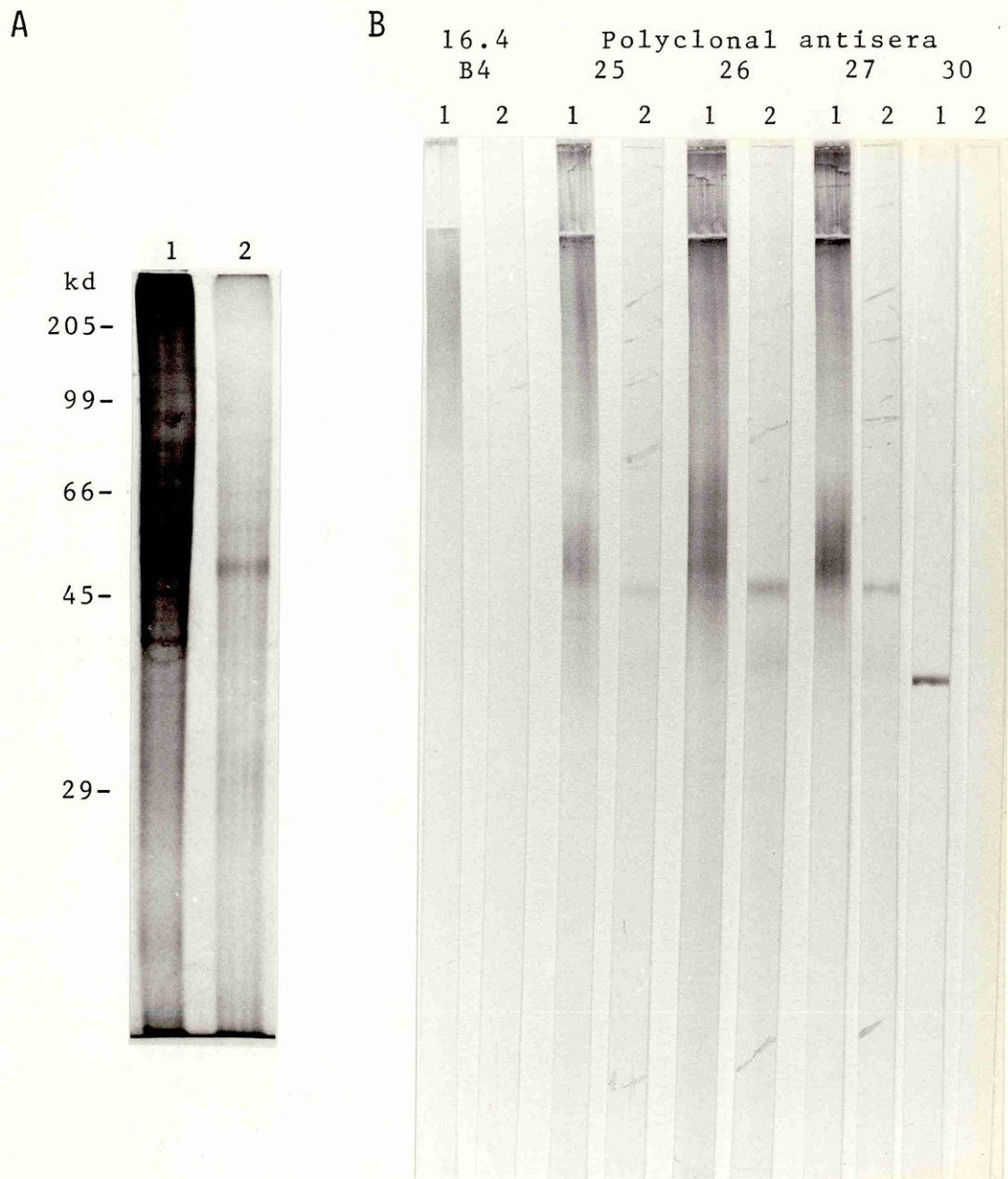


Figure 6.7. Silver stained gel and Western blot of glycosylated and deglycosylated proteins from *N. glutinosa*.

An SDS extract of *N. glutinosa* cells was size fractionated on Sephadex G-150, fractions containing 16.4B4 antigen pooled, extracted with isopentyl alcohol, and deglycosylated using trifluoromethane sulphonic acid. 1) glycosylated extract; 2) deglycosylated extract. A) silver stained gel; B) Western blot-antibody probes are indicated.

That this represents the deglycosylated form of the 16.4B4 antigen is indicated by the experiment shown in Figure 6.7B, in which deglycosylated and non-deglycosylated samples are probed on Western blots with polyclonal antisera to the 16.4B4 antigen. Deglycosylation abolished binding of monoclonal antibody 16.4B4. Whereas the polyclonal antisera reacted with the characteristic disperse bands in the non-deglycosylated sample, such reaction was not evident in the deglycosylated sample, but was replaced by binding to the prominent discrete band seen on the silver stained gel shown above (Fig. 6.7A). As expected, monoclonal antibody 16.4B4 did not react with the deglycosylated sample. When the monoclonal antibodies raised against the purified 16.4B4 antigen were tested for their reactivity against the deglycosylated sample, only clone 27.1E8 showed reactivity, which was directed against the 50 kd protein (result not shown). Polyclonal antisera against the 17.3A6 antigen also reacted with the non-deglycosylated sample, underlining the molecular weight heterogeneity of the starting material, but did not react with the deglycosylated sample.

#### Amino acid analysis.

The yield of protein for amino acid analysis following immunoaffinity chromatography and gel filtration was 16 ug. The positions of elution from the column of amino acids from the hydrolysed protein were compared to those of samples of the pure amino acids, to identify the eluted peaks. The composition of the 16.4B4 antigen determined by this analysis is shown in Table 6.1. The ratio of OD<sub>570</sub> to OD<sub>405</sub> for the ninhydrin adduct is characteristically different for proline and hydroxyproline from that for other amino acids (Robinson, 1978). This ratio did not deviate significantly from the expected value for all amino acids except aspartate, for which the ratio was atypical. It was found that a pure sample of hydroxyproline coeluted with aspartate. Thus the protein must contain a proportion of hydroxyproline residues, although since these two amino acids were not resolved, the mole percentage could not be calculated.



Table 6.1. Amino acid analysis of purified 16.4B4 antigen.

Amino acid	Mole percent
Alanine	13.9
Arginine	2.1
Aspartate *	8.4
Cysteine	0.6
Glutamate **	11.5
Glycine	14.7
Histidine	0.1
Isoleucine	3.0
Leucine	5.1
Lysine	1.4
Methionine	0.9
Phenylalanine	2.2
Proline ***	4.7
Serine	16.2
Threonine	7.8
Tryptophan	Not detected
Tyrosine	1.8
Valine	5.5

\* Asparagine, aspartic acid and hydroxyproline-not resolved.

\*\* Glutamine and glutamic acid-not differentiated.

\*\*\* Not including hydroxyproline.

Antigen levels during the culture period.

Fig. 6.8 shows the time course for the accumulation of the 16.4B4 antigen during the growth of the culture; Fig. 6.9 is a similar time course for the 17.3A6 antigen. The 16.4B4 antigen shows marked accumulation toward the end of the culture period, on days 12, 13 and 14, whereas the 17.3A6 antigen accumulates to the highest levels per unit fresh mass from day 4 to day 9. In addition, monoclonal antibody 16.4B4 binds to a low molecular weight discrete band, which is present in high amounts at the beginning of the culture period, but decreases during the middle of the cycle, increasing again toward the end. In contrast, 17.3A6 polyclonal antiserum reacts exclusively with the band of 36 kd on the blot.

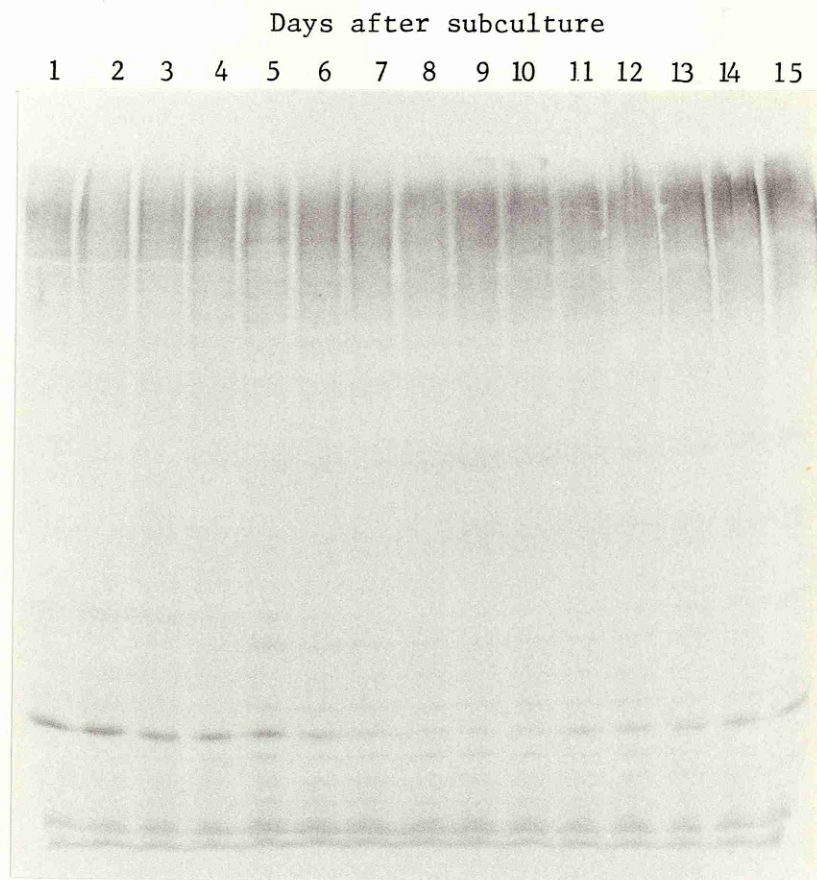


Figure 6.8. 16.4B4 antigen levels during growth of the *N. glutinosa* culture.

Cells from culture were frozen in liquid nitrogen on successive days after subculture. 2 g of cells from each time point were homogenised in 2 ml of SDS-PAGE sample buffer, and a 5  $\mu$ l aliquot resolved by SDS-PAGE. Proteins were immobilised on nitrocellulose, and probed with 16.4B4 monoclonal antibody.

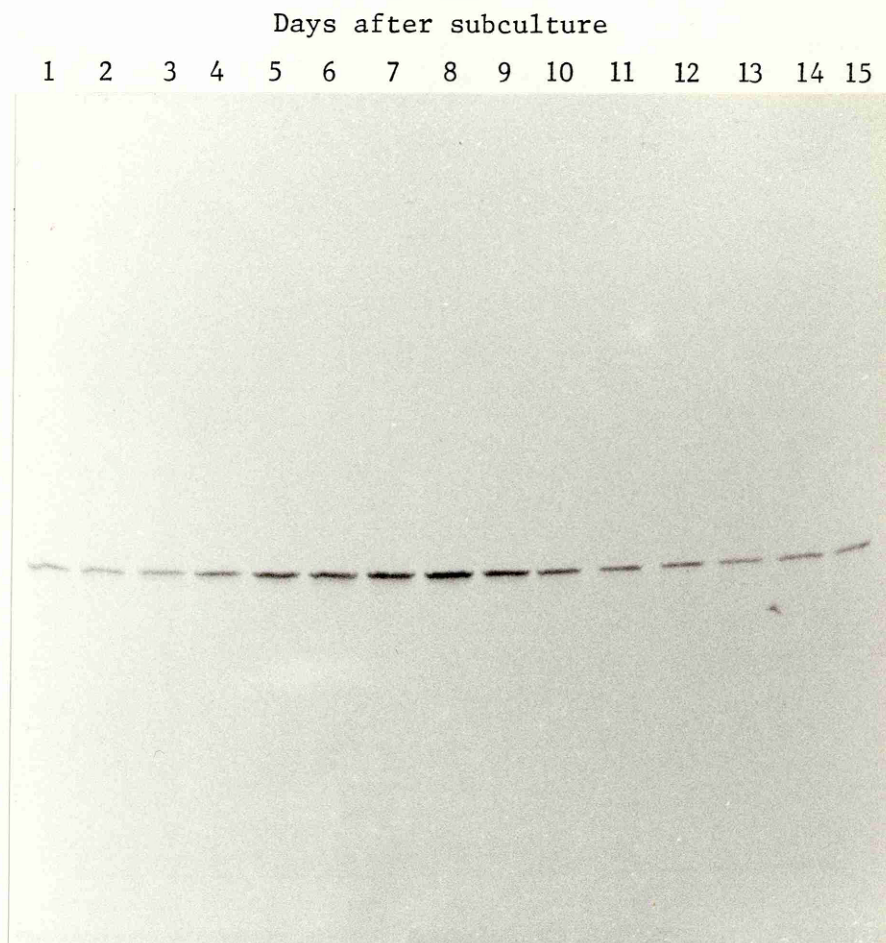


Figure 6.9. 17.3A6 antigen levels during growth of the N. glutinosa culture.

Conditions were as for Fig. 6.8, except that the probe was 17.3A6 polyclonal antiserum.

## DISCUSSION.

### Immunoaffinity chromatography.

Immunoaffinity chromatography of detergent extracts of *N. glutinosa* cells was found to be a rapid, highly selective method of isolating the proteins recognised by antibodies 16.4B4 and 17.3A6 in essentially pure form in a single step. The choice of detergent was important in successful execution of this procedure. The 17.3A6 antigen could be extracted from cells by treatment with buffer alone (Fig. 6.6B), but was extracted more effectively by the inclusion of NP-40 or CHAPS in the extraction medium. These detergents solubilise this antigen more effectively than boiling in SDS solution, which is considered to extract essentially all cellular protein into solution, by denaturation of secondary structure, and stabilisation as a detergent bound random coil (Helenius and Simons, 1975; Tanford and Reynolds, 1976). SDS extraction would be inappropriate for immunoaffinity chromatography of the 17.3A6 antigen, since the antibody fails to recognise the protein after denaturation, due to the lability of the recognised epitope. Since TBS alone will solubilise the 17.3A6 antigen, it appears that this protein is only weakly associated with membranes, suggesting that it is a peripheral protein, or maybe associated with a peripheral protein. Immunofluorescence microscopy of fixed and permeabilised protoplasts indicates that this protein is a cytoskeletal element, which may be anchored to the plasmalemma, and other membranes, as is the case for such elements in animal cells (results of M.S. Fitter; not shown). The monoclonal antibody was first recognised by its binding to total cellular membranes in radioimmunoassay, and the antigen must also have been present in the membranes with which the mice were initially immunised. Thus at least a proportion of the antigen must be fairly strongly attached to cellular membranes, or present as polymers with sufficient mass to be pelleted by low speed centrifugation, in order that they be repeatedly pelleted during the radioimmunoassay procedure.

Very little of the 16.4B4 antigen is solubilised by TBS, and the amount solubilised by SDS is considerably in excess of that rendered soluble by CHAPS or NP-40. Thus it would appear that the bulk of the protein is intimately associated with cellular membranes. Peripheral proteins are extractable by high salt treatment (Singer, S.J. and Nicolson, 1972), and non-denaturing detergents such as CHAPS have been reported to be effective in solubilising many integral proteins from animal cell membranes (Hjelmeland *et al.*, 1983). That boiling in SDS is required to efficiently extract the 16.4B4 antigen implies that it is an integral plasmalemma protein, and probably has a highly hydrophobic component, which requires denaturation and detergent stabilisation to remove it from the lipid bilayer.

Since effective solubilisation requires the presence of SDS, the protein is not removed in a form in which it will be bound by antibodies in solution. The SDS will tend to denature other proteins, inhibiting their biological activity. This has presented a problem for others investigating membrane proteins (R. Allen, Salk Institute; personal communication). Lazarides *et al.* (1984) reported a solution to this problem which has proved effective in other cases. The use of 1% (w/v) SDS solution allowed these investigators to solubilise spectrin effectively from the chicken optic system. By diluting the SDS to 0.2% (w/v) and supplementing it with 1% (v/v) Triton X-100, it was possible to maintain the spectrin in solution without impeding the binding of antibodies which recognise antigenic determinants insensitive to denaturation, which allowed immunoprecipitation of this protein.

A similar advantage may be reaped for the 16.4B4 antigen. Since it is recognised on Western blots, the epitope detected by the monoclonal antibody must be insensitive to denaturation, or renature when detergent is removed, during the antibody labelling of the antigen bound to nitrocellulose filters. Thus boiling in an SDS solution results in effective solubilisation of the antigen, whilst dilution of this solution with a Triton X-100 containing solution produces a milieu in which it will be bound by antibodies, and can be purified by

immunoaffinity chromatography. Since the antibody binds strongly and specifically to the 16.4B4 antigen, the large volume of diluted solution which must be passed down the column does not present a hindrance to purification. The antigen is concentrated by antibody binding, and so may be eluted in relatively few fractions once the pH of the eluent is reduced.

The silver stained gel and Western blot of the eluted material (Fig. 6.1) indicate that this approach has proved successful in the isolation of the 16.4B4 antigen in essentially pure form. Although the antigen is rather weakly stained by the silver procedure, it is clear that it is specifically eluted, and that there is little non-specifically bound material co-eluted. The Western blot indicates that the eluted protein does indeed represent the authentic 16.4B4 antigen. Of interest is the presence of a more intensely silver stained discrete band within the continuum of staining, which is not seen in the Western blot. The cause of this discrepancy is obscure. It is known that the efficiency of electroblotting is inversely related to the molecular weight of the protein, as this will determine its mobility in the applied electric field. In addition, the net charge of the protein at the pH at which blotting is performed (in this case, pH 9.3) will influence the mobility during transfer (Gershoni and Palade, 1983). It is unlikely that a protein of over 100 kd will be transferred quantitatively to the nitrocellulose filter. Poor transfer of the protein due to its high molecular weight may result in little additional transfer of the more intensely silver stained species over that of the rest of the antigen, and thus little or no additional Western blot reactivity of this molecular weight.

#### Antibody production.

The results of immunisation of mice with the purified antigen are radically different for the 17.3A6 and 16.4B4 antigens. In the former case, the polyclonal serum produced had a high titre against the antigen, and produced a strong signal on Western blots at 1:2000 or greater dilution. Since it has been shown (Chapter 5 and Fig. 6.4)

that 17.3A6 monoclonal antibody will not react with the 17.3A6 antigen on Western blots, due to the irreversible denaturation of the recognised epitope, the antibodies in the polyclonal antiserum binding to this molecule must be recognising other epitopes on the protein. The irreversible denaturation of the immunodominant epitope during the preparation procedure is an advantage in generating antisera of broader specificity, which will react with the antigen under a wider range of conditions. There was slight reactivity of the highest titre polyclonal antiserum against bands in the Western blot of lower molecular weight than the immunogen. It is possible that these represent weak cross-reactivity with other cellular proteins, but more likely, since they have molecular weights close to but slightly lower than the major band that they represent degradation products. It is entirely possible that during the extraction procedure a small amount of proteolysis occurs due to the release on homogenisation of proteases which are compartmentally isolated in the intact cell. That these bands represent a minor component of the total immunoreactivity in the gel suggests that extraction by homogenisation and boiling in SDS is an effective method of rapidly inactivating any proteases released.

The binding of polyclonal antiserum to the antigen is not periodate sensitive, suggesting that the antigen contains no carbohydrate component. That there is no cross-reactivity of the polyclonal antiserum with other *N. glutinosa* proteins tends to support this contention. There would be a greater likelihood that an epitope of a carbohydrate moiety of a glycoprotein would be common to several glycoproteins, than a proteinaceous epitope, since many glycoproteins can share a common core oligosaccharide (see Chapter 9), whereas there are no constraints on the permutations of amino acid residues in a polypeptide.

The difference between the antigenicity profiles expected for glycosylated and non-glycosylated proteins is amply demonstrated by the results of a similar immunisation regime using the 16.4B4 antigen. In this case, the reactivity pattern of the polyclonal antiserum was



highly complex (Fig. 6.3). In addition to a diffuse band in the gel of the same molecular weight distribution as that recognised by the monoclonal antibody, a lower molecular weight diffuse band and a series of more discrete bands were seen. The binding of the antibody to the diffuse bands showed periodate sensitivity, although this was not complete even at 50 mM periodate concentration, in contrast to the binding of the monoclonal antibody, which is abolished after 10 mM periodate treatment of the blot. The more discrete bands show more limited periodate sensitivity. There are also differences in reactivity pattern between mice. Whereas serum 25 recognises predominantly the immunising antigen, and a discrete band of 50 kd molecular weight, serum 26 shows more binding to the lower molecular weight diffuse band, and two discrete bands of 87 and 40 kd. Serum 27 shows a similar intensity of binding to both diffuse bands, as well as some binding to the 50 kd discrete band.

The situation for the monoclonal antibodies raised from these mice is even more complex (Fig. 6.5). Whereas some of the monoclonal antibodies (eg: 27.1E3) recognise a diffuse band of similar molecular weight distribution to the immunogen, others recognise in addition a diffuse band of lower molecular weight. Monoclonal antibodies 25.6D4 and 27.5A2, which recognise a series of low molecular weight discrete bands represent an enigma. In contrast, a series of antibodies react weakly with a 50 kd band on blots, which appears more discrete. This may be the same band as is recognised by the polyclonal antisera. Still other clones recognise other discrete bands of different molecular weights.

There are two possible explanations for this complexity. First, some of the bands could represent reactivity to contaminants in the immunising solution, or second, they could be the result of extensive homology between the 16.4B4 antigen and other cellular glycoproteins. Although the silver stained gel of the affinity purified antigen (Fig. 6.1A) shows no evidence of other proteins being coeluted from the column, it is possible that a trace amount of a contaminating protein could make a significant contribution to the composition of the

antiserum, if it is more antigenic than the 16.4B4 antigen. An additional problem is the difficulty in quantifying the amount of protein being injected. Because the 16.4B4 antigen requires detergent to maintain it in solution it cannot be quantified, as the detergents SDS and Triton X-100 both interfere with protein assays. The extensive glycosylation inferred from the periodate sensitivity and deglycosylation experiments (see below) appears to interfere with silver staining of the antigen, and might also impede quantification. Certainly the immune response against the 16.4B4 antigen was weaker than that against the 17.3A6 antigen. Whereas the latter is still recognised strongly by antiserum at 1:2000 dilution, colour development of the Western blot of the former is much slower, even at 1:250 dilution. This might represent a difference in amount of antigen administered to the mice or in the antigenicity of the different proteins.

The second explanation of the heterogeneity of the antisera and monoclonal antibodies against the 16.4B4 antigen, namely that other glycoproteins express the same epitope, certainly might be valid. The pattern of reactivity of monoclonal antibody 26.2E5 shows two diffuse bands, the higher molecular weight one comparable to the 16.4B4 antigen, and the other of lower molecular weight. This is similar to the two major components of reactivity of the polyclonal antisera, and also to the reactivity patterns of the clones Nt 3.2B4, Nt 4.2A4, Nt 4.3E5 and Nt 4.4E4, raised against *N. tabacum* protoplasts (Chapter 5). These monoclonal antibodies recognise not only the plasmalemma, but the cell wall and intracellular components, such as vacuoles (unpublished results of M. G. Hahn). It is concluded that these antibodies recognise a common carbohydrate constituent among a number of glycosylated proteins in plant cells. As indicated above, it is well known in many eukaryotic cells that a number of glycoproteins are glycosylated by a single pathway during maturation (Hubbard and Ivatt, 1981). The core oligosaccharide is transferred from dolichol phosphate, which acts as an oligosaccharide carrier (Sefton, 1977), to polypeptides to form the core glycosylated glycoprotein. A series of modifications to the core oligosaccharide occurs subsequently, to

yield the mature glycoprotein (Kornfeld and Kornfeld, 1985). A similar process may be responsible for glycoprotein formation in plant cells (Elbein, 1979). In this case, it would be expected that whereas monoclonal antibody 16.4B4 recognises an epitope unique to the mature protein, antibody 26.2E5 and the four monoclonal antibodies raised against *N. tabacum* would recognise a component of the common core of glycosylation of a number of similarly glycosylated glycoproteins (see also Chapter 9).

The epitopes recognised by the polyclonal antisera did not all show periodate sensitivity on Western blots (Fig. 6.3). Periodate effectively oxidises vicinal hydroxyl groups of sugar residues, without damage to polypeptides (Bobbitt, 1956). Not all carbohydrate epitopes are necessarily sensitive to this treatment, however, and it is possible that components of the polyclonal antisera recognise periodate insensitive sugars or linkages. Alternatively, the periodate insensitive component of the reactivity might be directed against proteinaceous epitopes. It is clear from the proteinase K treatment recorded in Fig. 6.6C that the 16.4B4 antigen contains a protein component. Extraction of the protein from the plasmalemma and denaturation by boiling in SDS will open up the structure of the glycoprotein, and expose epitopes not presented to the immune system in the membrane bound form in which the protein was injected into mice in the initial set of fusion experiments, either due to their being buried in the structure, or intercalated in the plasmalemma. It is plausible that the periodate insensitive antibody reactivity is directed against such epitopes. Nevertheless, the major component of the antibody reactivity appears to be directed against periodate sensitive epitopes. Whereas the purification procedure for the 17.3A6 antigen denatures the immunodominant epitope, the purification of the 16.4B4 antigen does not remove the carbohydrate epitope which is apparently immunodominant in the native protein. Thus it is not surprising that a high proportion of the reactivity of the polyclonal antiserum is again directed against a periodate sensitive epitope.

Perhaps less expected are the number of monoclonal antibodies raised against the purified 16.4B4 antigen which show little or no binding in Western blots to material of the same molecular weight as the immunogen, but instead react with lower molecular weight species. It might be concluded from such an observation that the eluted material was contaminated with proteins non-specifically bound to the column, as postulated above. Another possible explanation would be that the immunogen expresses with low frequency epitopes, such as carbohydrate residues or polypeptide conformations, exhibited at much higher frequencies by other proteins. Thus antibodies raised against such epitopes would preferentially bind to proteins other than the immunogen. Such an effect is masked in polyclonal antisera by the heterogeneity of epitope specificities of antibodies generated. Thus, even if antibodies of broad cross-reactivity were generated, they might represent such a small component of the total epitope specificity of the polyclonal antiserum that their cross-reactivity would not be detected above the background. In contrast, hybridoma production selects a single antibody specificity from among the many generated in response to an antigen, and so can potentially isolate such atypical specificities. Such a mechanism has been proposed to explain the observed cross-reactivity of certain monoclonal antibodies with apparently unrelated proteins (Lane and Koprowski, 1982; Dulbecco et al., 1981).

#### Chemical deglycosylation.

The results of the chemical deglycosylation experiment go some way towards resolving the complexities of the antibody response to the 16.4B4 antigen. Deglycosylation with trifluoromethane sulphonic acid represents the most satisfactory of a number of such procedures. Enzymatic deglycosylation can be a highly effective method, certain enzymes removing all saccharide moieties from appropriate substrates (Plummer et al., 1984). However, many of the enzymes have rather stringent requirements as to the oligosaccharide chain length and composition necessary for activity (Kobata, 1979). It is not known whether the composition of the oligosaccharide components of the

16.4B4 antigen satisfy such requirements. Anhydrous hydrogen fluoride was introduced by Mort and Lamport (1977) as an effective agent for the deglycosylation of glycoproteins, but is difficult to work with and requires specialised equipment. The chemical deglycosylation method of Edge *et al.* (1981) provides a satisfactory alternative. Although most side chain oligosaccharides were rapidly and efficiently removed from glycoproteins, these authors found that N-glycosidically linked N-acetylglucosamine residues were resistant to hydrolysis. Thus the band of 50 kd seen in silver stained gels after deglycosylation (Fig 6.7A) may not represent the completely deglycosylated core polypeptide, but has probably been stripped of much of its glycosylation. This treatment has resulted in loss of molecular weight heterogeneity of the protein, indicating that this is the result of microheterogeneity of glycosylation, as previously postulated (Chapter 5). There is an apparent decrease in molecular weight of the antigen of 80-180 kd upon deglycosylation. Although this cannot be concluded to represent the real molecular weight contribution of the carbohydrate portion of the glycoprotein, since carbohydrate moieties will alter the charge:mass ratio and hence the mobility of the protein in polyacrylamide gels (Grefrath and Reynolds, 1974), it nevertheless indicates that the 16.4B4 antigen is highly glycosylated.

Interestingly, although from Western blots of non-deglycosylated protein (Fig. 6.7B) there is a considerable amount of material from the lower molecular weight diffuse band present in the sample, after deglycosylation only a single discrete band of reactivity with the polyclonal antiserum is seen on the gel. This suggests that the two diffuse bands may share a common core polypeptide, but differ in the amount, pattern or net charge of glycosyl moieties linked to this core. It is possible that a preliminary round of glycosylation generates the lower molecular weight band, which is subsequently modified to produce the mature higher molecular weight species recognised by monoclonal antibody 16.4B4.

## Amino acid analysis.

There are a number of caveats to be considered in the interpretation of the amino acid analysis results. The use of glycine to elute the bound 16.4B4 antigen from the immunoaffinity column could have led to overestimation of this amino acid in the analysis, but the extensive dialysis, gel filtration and solvent extraction procedures subsequently undertaken are likely to have reduced this possibility. There was strong evidence of hydroxylation of some of the proline residues of the protein, as judged by the absorbance ratio of the Asx peak in the analysis, although the method of amino acid analysis used did not allow the amount of hydroxyproline to be calculated. Tryptophan residues are destroyed by acid hydrolysis, and so cannot be resolved by this method, whilst cysteine residues are susceptible to degradation by concentrated hydrochloric acid, which is the usual hydrolytic reagent. The use of methane sulphonic acid to hydrolyse the protein reduces the extent of degradation of the latter amino acid, leading to its more accurate quantification.

Despite these potential problems, a number of conclusions may be reached from this analysis. There is a high serine, threonine and hydroxyproline content in the protein, all of which are potential sites of O-linked glycosylation of the peptide. In addition, there is the potential for N-linked glycosylation via asparagine. Hexosamines interfere with amino acid composition analyses, since they are eluted from the column at a comparable salt concentration to tyrosine residues, and react with ninhydrin (Oldberg *et al.*, 1981). No hexosamine peak was detected in the analysis, indicating that such sugars were not present in the 16.4B4 antigen. Other conclusions from the results of the amino acid analysis will be discussed in Chapter 9.

## Antigen levels during the culture period.

The results of the experiments on the time course of accumulation of the 16.4B4 and 17.3A6 antigens may best be interpreted with regard to Fig. 2.1, which shows the cell mass increase and other culture

parameters over the same time course. The 16.4B4 antigen increases in amount at the time when the culture is entering the stationary phase of the growth cycle, and is less represented during the phase of active growth. Thus, it would appear that an increased synthesis of this protein is a response to the cessation of active growth.

In contrast, the 17.3A6 antigen is present in greatest amount in cells immediately prior to, and during the initial part of the phase of rapid growth. As was indicated above, this protein is probably a cytoskeletal element, so that its increased synthesis before and during the initial period of growth may presage the onset of cell division, which requires an increased rate of mitosis and cytokinesis-both processes which involve the cytoskeleton. In intact plants, cell division is accompanied by little increase in size of the combined daughter cells over the parental cell, but is followed by a period of growth of cells by expansion. Such a process may also occur in suspension culture cells. If this is happening in the N. glutinosa culture, the period of maximum expression of the 17.3A6 antigen may correspond to that of maximum rates of cell division. The subsequent increase in mass of the cells in culture would then be a result predominantly of cell expansion, rather than cell division. This would explain the decreasing expression of the 17.3A6 antigen later in the growth cycle. When stationary phase is reached, the amount of antigen expression is substantially decreased.

Heim et al. (1985) found a similar increase in expression of protein kinase activity at the onset of the period of rapid growth of N. tabacum suspension cultures, with a subsequent decrease in activity later during active growth. As here, they concluded that a phase of cell division in culture is followed by a phase of growth by expansion.



PLANT PLASMALEMMA STRUCTURE:  
AN IMMUNOLOGICAL APPROACH

PAUL MICHAEL NORMAN, PhD

JULY 1986

PART 3 OF 3

CHAPTER SEVEN.

SUCROSE DENSITY GRADIENT CENTRIFUGATION.



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## INTRODUCTION.

Whereas - the previous chapters concerned the greater characterisation of the antigens recognised by the monoclonal antibodies secreted by the hybridoma library, this chapter deals with the use of these antibodies as markers in detection of the plasmalemma in cell homogenates. In Chapter four, it was demonstrated that hybridomas from the library secreted antibody recognising plasmalemma whilst others probably recognise intracellular structures. It is well documented in both plant (Robinson, 1985) and animal (Wallach and Lin, 1973) cells, that different membranes have different buoyant densities in sucrose density gradients. Distribution of the membranes has typically been assessed by the use of marker enzyme activities, thought to be characteristic of certain membranes (see Chapter 1).

Total cellular membranes were isolated from N. glutinosa cells, and resolved by isopycnic centrifugation on sucrose density gradients. Using a slot blot assay in which aliquots of fractions from such a gradient were immobilised on nitrocellulose and probed with various monoclonal antibodies, followed by iodinated second antibody, it was possible to detect fractions enriched in specific antigens. The distribution of antigenicity differed for internal epitopes and plasmalemma-expressed ones, although the latter were found to show a rather complex distribution. A second assay simplified the interpretation of these results. When intact protoplasts were labelled with surface reactive antibody prior to membrane fractionation, it was possible to exclude intracellular binding sites. Such "topologically specific" labelling resulted in a single peak of antibody binding in the density gradient. Parallel experiments with disrupted protoplasts and with an antibody reactive with an intracellular antigen were useful controls, which demonstrated the antibody and plasmalemma specificity of the technique.

## MATERIALS AND METHODS.

### Density gradient centrifugation.

40 g of cells from *N. glutinosa* suspension cultures 4-6 days after subculture were ground in homogenisation buffer at 4°C in a pestle and mortar, as described in Chapter 2. After centrifugation at 100 000  $g_{av}$  for 1 h, the pellet was resuspended in 1-2 ml of homogenisation buffer and layered onto a 15-45% (w/w) sucrose density gradient. This was made in 35 ml of 2.5 mM Tris-MES, 1 mM dithiothreitol, 0.5 mM EDTA, pH 7.4, in a 37.5 ml ultracentrifuge tube. Tubes were centrifuged in a Beckman SW28 rotor at 100 000  $g_{av}$  and 4°C overnight (15-18 h), with slow acceleration and deceleration without braking. After centrifugation, the gradient was separated at 4°C into 24 fractions of 30 drops (ca. 1.5 ml) using an LKB fraction collector, by sinking a capillary into the centrifuge tube and withdrawing fractions from the bottom. Sucrose content for each fraction was measured at 25°C using a 40  $\mu$ l aliquot in an Abbe refractometer. The rest of the material was kept on ice until required.

### Slot blot assay.

A nitrocellulose filter (4.5  $\mu$ m pore size, Schleicher and Schuell, Keene, New Hampshire) and two sheets of filter paper were pre-wetted with distilled water, and placed in a slot blot manifold (Schleicher and Schuell), care being taken to avoid trapping air between the layers. The filter was dried by drawing air through the manifold for at least 5 min using a water aspirator pump.

An aliquot of each fraction from the density gradient was diluted 5- or 10-fold with PBS, and 20  $\mu$ l of diluted material applied to each slot of the manifold. The nitrocellulose filter was dried as above for 5-10 min and then blocked for 30 min with 135  $\mu$ l/well of 5% (w/v) BSA in PBS. The blocking agent was removed by aspiration, and 40  $\mu$ l/well of undiluted hybridoma supernatant applied for 1 h at room temperature. This was aspirated, the filter removed from the manifold

and incubated for 30 min by agitation on a rocker platform in three changes of PBS containing 0.05% (v/v) Tween 20. Remaining protein binding sites on the filter were blocked by incubation for 30 min in 5% (w/v) BSA in PBS, before incubation with agitation in 2  $\mu$ l/ml  $I^{125}$ -labelled rabbit anti-mouse antibody in the same solution. The filter was washed for 1 h in 5-6 changes of PBS-0.05% (v/v) Tween 20, dried and autoradiographed overnight using Kodak XRP-1 film with a Cronex intensifying screen at  $-70^{\circ}\text{C}$ . Films were developed using a Kodak M20 processor.

Intensity of silver deposition for individual slots was assessed using a Beckman DU8-B spectrophotometer equipped with a gel scanning accessory and gel scan module. Sensitivity and chart speed were chosen such that peaks when recorded had a small base:height ratio, and peak height could be used as a measure of intensity of silver grain deposition. Peak height was plotted against fraction number.

Topologically specific labelling.

Ca.  $4 \times 10^7$  *N. glutinosa* protoplasts were produced from 32 g of suspension culture cells in 300 ml of enzyme solution as described in Chapter 2. The washed protoplasts were divided into four parts and treated as below:

i) Protoplasts were incubated with osmotically balanced 16.4B4 hybridoma supernatant and washed as described for immunofluorescence labelling (Chapter 4), then incubated for 45 min at  $4^{\circ}\text{C}$  in 50  $\mu$ l of  $I^{125}$ -labelled rabbit anti-mouse antibody diluted 1:20 in 0.4 M sorbitol, 0.1% (w/v) HEPES, 1 mg/ml BSA, 0.1 mg/ml goat gammaglobulins (IF wash). The protoplasts were washed three times with 5 ml of IF wash, then lysed in homogenisation buffer using a glass homogeniser. Debris was removed by centrifugation at 1000 g for 10 min, and membranes pelleted at  $100\,000\ g_{av}$  for 1 h. The pellet was resuspended in 1 ml of homogenisation buffer and loaded onto a 15-45% (w/w) sucrose density gradient of composition and subsequent treatment as described, except that it was fractionated to 37 fractions of 20

drops.

ii) Protoplasts were treated as above, except that the first antibody was from 17.4C5 hybridoma supernatant.

iii) Protoplasts were lysed in IF wash using a glass homogeniser, prior to incubation for 45 min with 16.4B4 hybridoma supernatant. The homogenate was washed by pelleting once from 10 ml of IF wash at 100 000  $g_{av}$  for 1 h. The pellet was resuspended using a teflon-glass homogeniser and incubated with  $I^{125}$ -labelled rabbit anti-mouse antibody as described in (i) above. After 45 min, the homogenate was diluted to 10 ml with homogenisation buffer, debris removed by centrifugation at 1000 g for 10 min, and membranes pelleted at 100 000  $g_{av}$  for 1 h. The pellet was resuspended and layered onto a sucrose density gradient as described above.

iv) Protoplasts were treated as in (iii), except that the supernatant was from hybridoma 17.4C5.

Radioactivity in each fraction from each gradient was determined by gamma-counting, sucrose content was determined as above.

## RESULTS.

Parameters of the slot blot assay.

It was necessary to dilute fractions at least 5-fold with buffer before application to nitrocellulose filters, to avoid a high background of non-specific antibody binding, presumably due to the high sucrose content of the undiluted samples. 20  $\mu$ l was the minimum volume which would reliably wet the entire surface of the filter in a single slot, but it was found that larger volumes could be applied to the slots without detriment.

The gradient fraction showing the maximum antibody binding (see below) was selected to test the effect of dilution of the antigen on the slot blot signal for several antibodies. The material in the fraction was diluted 1:10 with PBS, then further as indicated, before application to nitrocellulose sheets. Each dilution was applied in triplicate, and the extent of antibody binding assessed by the peak height on autoradiograms measured as described above. Fig. 7.1 shows the result of this determination for clones 16.2C6, 16.4B4 and 17.4B5.

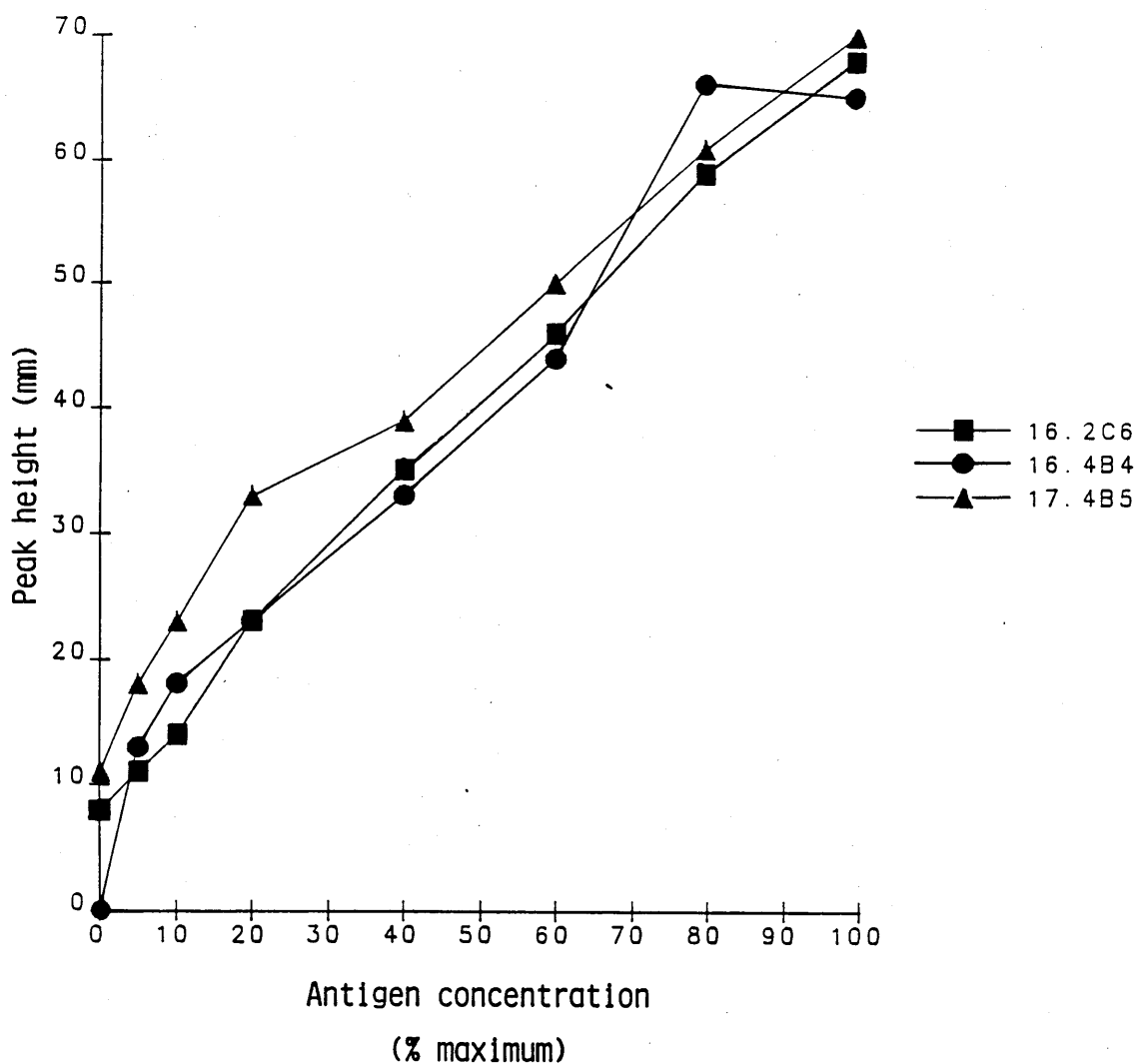


Figure 7.1. Antigen concentration dependence of the slot blot assay.

Fraction 21 from a sucrose density gradient was diluted 1:10 in PBS, then to 5, 10, 20, 40, 60 and 80% of this concentration. Diluted fractions were applied to nitrocellulose filters in a slot blot manifold, and probed with hybridoma supernatants and  $I^{125}$  labelled anti-mouse antibody as described. Autoradiographs of filters were scanned, and peak height determined and graphed.

## Assay of monoclonal antibody binding to gradient fractions.

When the binding capacity for various monoclonal antibodies of fractions from density gradients was assessed, clear differences between fractions were seen (Fig. 7.2). It was found that the surface reactive monoclonal antibodies (as assessed by immunofluorescence microscopy) all showed a similar pattern of binding, with two peaks of reactivity, a sharp one at 1.08 kg/l, the other being broader, and peaking at 1.17 kg/l. In contrast, non-surface reactive antibodies showed different patterns of reactivity, both from the surface reactive antibodies and from each other. Several of the non-surface reactive clones (17.3A3, 23.1D5; results not shown) showed no clear binding maxima within the gradient, in contrast to 17.3A6 and 17.4C5, for which the maxima were at 1.10 kg/l and 1.11 kg/l respectively.

The surface reactive antibodies showed rather high levels of binding throughout the gradient, suggesting that there was some dispersity in density of membranes carrying the antigen. In particular, the shallow slope of the less dense side of the higher density peak is suggestive of this phenomenon. In contrast, the peak density for the 17.3A6 antigen was sharply defined, with other fractions showing only 10-20% of the peak value. There was also considerable heterogeneity in density of fractions expressing the antigen recognised by antibody 17.4C5, with a slight shoulder at higher densities, and a minor peak of reactivity roughly coincident with the higher density peak of the surface expressed antigens. The pattern of reactivity for all of the clones differed from the distribution of protein content, as assessed by OD<sub>280</sub> measurement of an aliquot of each fraction (not shown).



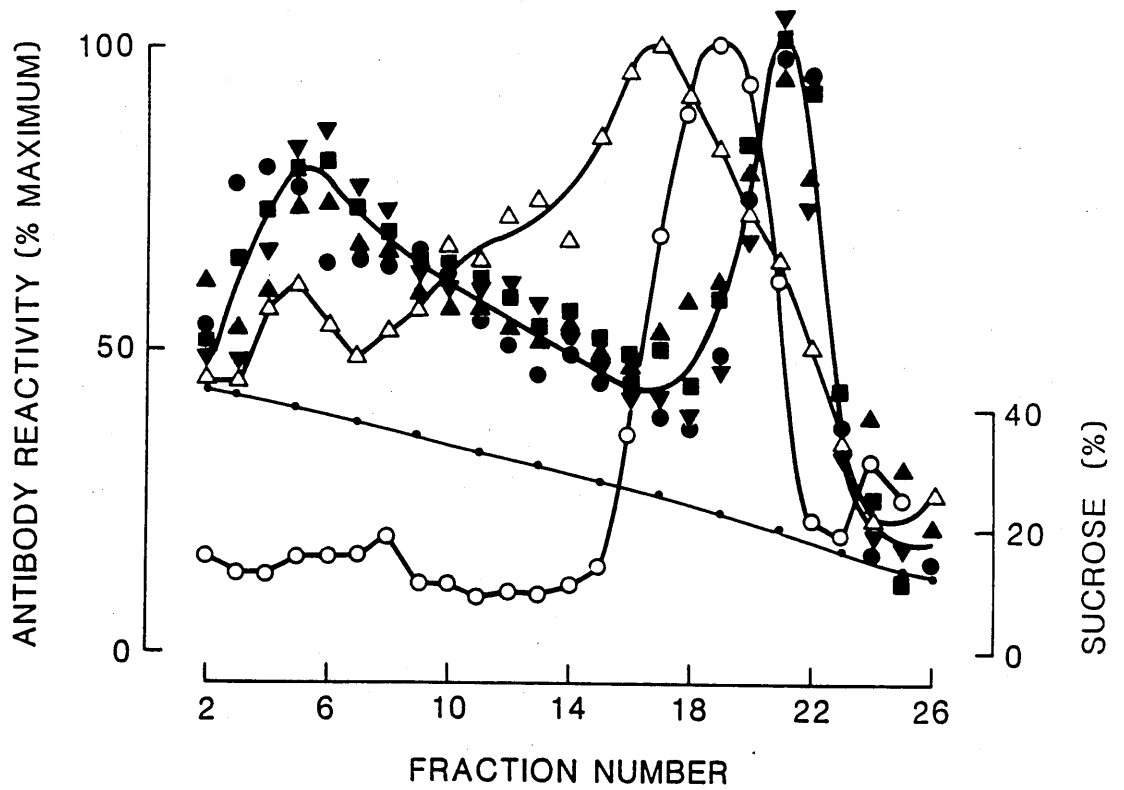


Figure 7.2. Results of slot blot assay.

Distribution of antigenic material within the gradient was determined by a slot blot as described.—●—: gradient profile. Monoclonal antibody probes were: —■—: 16.2C6, —▼—: 16.4A6, —●—: 16.4B4, —○—: 17.3A6, —▲—: 17.4B5 and —△—: 17.4C5. Antibodies reactive with the plasmalemma are denoted by closed symbols, those reactive with intracellular antigens by open symbols.

### Topologically specific labelling.

Figure 7.3 shows the results of labelling of protoplasts and protoplast homogenates. Panel A shows the density distribution of membrane bound label when protoplasts were labelled with monoclonal antibody 16.4B4 and iodinated second antibody prior to homogenisation. The labelling is clearly specific for one broad peak in the gradient with maximum density of 1.14 kg/l. This is confirmed in Panel B, which shows the results from a separate experiment in which binding of antibody 16.4B4 to intact protoplasts was compared to binding to a protoplast homogenate. The extent of binding to the intact protoplasts was considerably less than that to the homogenate, but showed a clear maximum in the same region as seen in Panel A. In Panel B there is some evidence that the peak of binding to protoplasts is bimodal. The bulk of the additional binding of antibody 16.4B4 in protoplast homogenates was to the low density peak seen in Fig. 7.2. The amount of radioactivity measured in the higher density peak was similar for intact protoplasts and homogenates.

This contrasts with the results using antibody 17.4C5, which binds to an internally expressed epitope. In this case (Panel C), there was relatively little binding to intact protoplasts. This showed a slight maximum at ca. 1.12 kg/l, but no maximum in the position seen for 16.4B4 on protoplasts. There was substantially enhanced antibody binding to protoplast homogenates. This showed a maximum at 1.12 kg/l and a second at 1.05 kg/l, coincident with that seen for 16.4B4.

The open triangles in Figure 7.3 Panels B and C plot the ratio of reactivity in intact protoplasts to protoplast homogenates. There was clear selectivity in the binding of 16.4B4 to intact protoplasts for the higher density peak, with low ratios of reactivity for the lower density peak. In contrast, for 17.4C5 there was little selectivity in binding to protoplasts relative to protoplast homogenates. The antibody reactivity ratio remained low throughout the gradient.

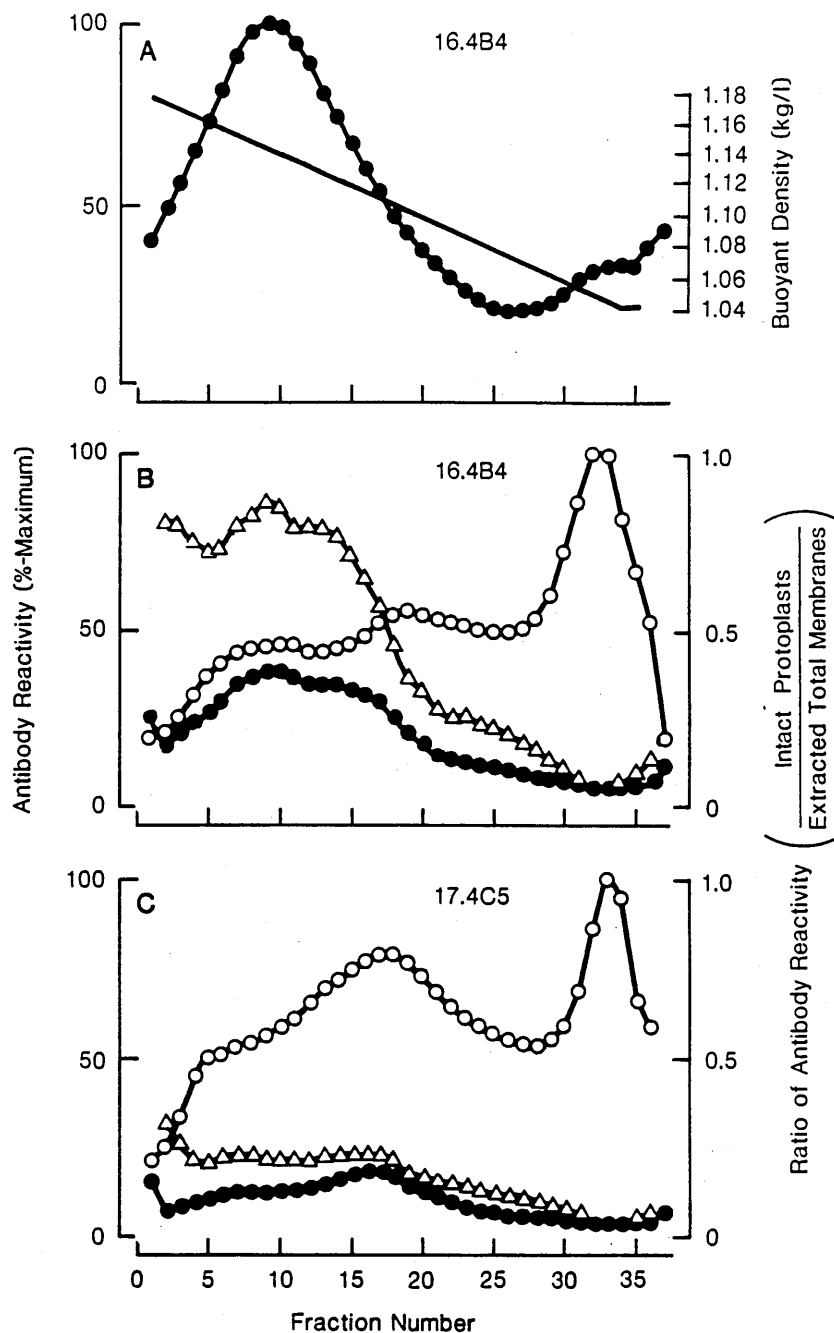


Figure 7.3. Topologically specific labelling of protoplasts.

Protoplasts or protoplast homogenates were labelled with monoclonal antibody and radiolabelled second antibody. A and B: —●— labelling of intact protoplasts with 16.4B4 supernatant. B: —○— labelling of homogenised protoplasts with 16.4B4 supernatant. C: —●— labelling of intact protoplasts and —○— homogenised protoplasts with 17.4C5 supernatant. B and C: —△— ratio of reactivity of antibodies with intact protoplasts and homogenised protoplasts. A: ——— buoyant density of fractions.

## DISCUSSION.

Slot blot assay parameters.

The titration of antibody reactivity reported in Figure 7.1 suggests that the slot blot assay is approximately linear with respect to antigen concentration in the range in which it is used here. The absolute values of peak height differ for the different antibodies used; this effect is compensated for by use of percent maximum plots in Figure 7.2. Some non-linearity is seen at low antigen concentrations, but this is unlikely to be a problem in this study, since quantification is not attempted. It will, however, lead to overestimation of the amount of antigen present in non-peak fractions.

The investigation of antigen dependence of antibody reactivity reported here does not take account of the differences in membrane composition between different fractions. If a particular membrane were to show non-specific binding of the monoclonal antibody, this would alter the distribution profile seen in an homogenate.

A similar assay of antibody binding to proteins from rat brain synaptic vesicles immobilised on nitrocellulose has been reported by Jahn *et al.* (1984). They concluded that their assay was substantially linear over a 20- to 50-fold concentration range, in support of the results described here.

Slot blot assay.

The slot blot assay of gradient fractions shows that there are clear differences in the density distribution of membranes carrying epitopes reactive with different monoclonal antibodies. The four surface reactive monoclonal antibodies all show the same distribution, with two peaks of reactivity. This might be expected, however, since immunoblot analyses indicate that three of four bind to the same antigen (Chapter 5). There is a clear difference between this pattern and that seen for the two internally expressed antigens, which are

themselves non-contiguously distributed. Thus the slot blot assay allows differentiation of plant cell membranes based on their differential expression of antigenic determinants.

The antibody reactivity on slot blots for plasmalemma specific antibodies is clearly bimodal. Whereas the peak of 1.08 kg/l was sharp, that of 1.17 kg/l was rather broad, the slope being particularly shallow towards lower densities. The slot blot experiments do not define if one or both of these peaks represent the plasmalemma. It is interesting that the antigen recognised by antibody 17.4C5 also shows rather heterogeneous equilibrium density. This implies that both this antigen and the surface expressed epitopes are exhibited on more than one type of membrane. In contrast, the 17.3A6 antigen is of homogeneous density, indicating that it is uniquely associated with a single membrane.

#### Topologically specific labelling.

The labelling of intact protoplasts prior to disruption and fractionation has several major advantages over the slot blot assay, where antibody labelling occurs after fractionation. First, for all intact protoplasts in the preparation, labelling of intracellular structures is excluded. Thus, apart from any binding to internal membranes of leaky protoplasts, only surface accessible sites are expected to be labelled. By performing the labelling at 0°C, any active processes of membrane recirculation and internalisation of the antibody should be circumvented (Chapter 4). Second, antibody binding to the plasmalemma is detected regardless of the topology of vesicles formed upon homogenisation, since binding occurs prior to disruption. For the slot blot assay, it is likely that only antigenic determinants displayed on right side out or leaky vesicles, or membrane sheets are available for antibody binding. These differences between the assays may explain the different results obtained in each case.

The topological labelling results in only a single density peak when applied to the binding of the surface reactive antibody to intact

protoplasts (Fig. 7.3A). The peak is quite broad, occupying roughly 50% of the gradient, but shows unimodal distribution, suggesting that only one membrane species is recognised, even if this does show considerable density heterogeneity. There is some increase in antibody reactivity towards the top of the gradient, but this may simply represent particles too small to enter the gradient and which are occluded on the sample-gradient interface (Galbraith and Northcote, 1977). A similar pattern is seen for intact protoplasts in Fig. 7.3B, although the labelling does not show a single density peak, but a plateau of maximal binding. In contrast, antibody 17.4C5 shows a low level of binding in all gradient fractions when incubated with intact protoplasts. There is a slight peak of binding at a density of ca. 1.12 kg/l, which is close to the peak density seen for labelling by this antibody in the slot blot assays, and of the same density as one of the two major peaks seen for the binding of this antibody to protoplast homogenates. These results imply that a membrane of this density is the major site of expression of the 17.4C5 antigen, and that this is intracellular, and not accessible for antibody binding in intact protoplasts, confirming the results of immunofluorescence microscopy (Chapter 4). There is a small amount of binding of antibody 17.4C5 to membranes from intact protoplasts with the same density as the peak seen with 16.4B4. This may represent limited non-specific binding of the former antibody to the plasmalemma, and suggests that binding of 16.4B4 to intact protoplasts is antigen specific.

In contrast to its labelling of intact protoplasts, the labelling of protoplast homogenates with 16.4B4 is predominantly of membranes fractionating at lower densities. The large peak seen in this curve (Fig. 7.3B) appears to correspond to that seen in slot blot analyses, but has rather different characteristics. First, it represents a much larger proportion of the total reactivity in the gradient than that seen in slot blots. The higher density peak in the slot blot is not pronounced in the labelling of homogenates. Second, whereas this peak has a density of 1.08 kg/l in the slot blot analysis, its density when labelling homogenates is roughly 1.05 kg/l. 17.4C5 also shows a peak of this density when labelling homogenates, but does not on slot

blots. Either this represents a membrane fraction showing non-specific binding of antibodies, or occluded membranes or non-membrane bound material. Binding of 16.4B4 to protoplast homogenates also shows a peak at ca. 1.11 kg/l, similar to the major peak of 17.4C5 binding.

Although binding of 16.4B4 to membranes in protoplast homogenates of the peak density seen for binding to intact protoplasts is a small component of the total reactivity, the ratio of protoplast:homogenate binding is approximately 90% at this density. The two protoplast populations were treated differently, and so a direct comparison may not be valid. Whereas the intact protoplasts are liable to loss by lysis and non-pelleting during the repeated washes which they undergo, the homogenates are subjected to limited blocking and washing steps. Nevertheless, that the amount of antibody bound to membranes of this density is similar in both cases might suggest that almost all available plasmalemma binding sites are labelled in the intact protoplasts, and that these are largely exhibited on the external face of the plasmalemma. The ratio of binding to protoplasts relative to homogenates is low for membranes of density below ca. 1.12 kg/l. It is concluded that the peaks of binding to lower density membranes represent intracellular binding sites inaccessible to antibody in intact protoplasts. In contrast, the ratio of binding of antibody 17.4C5 remains low throughout the gradient, implying that there is no selective binding to surface exposed sites of the protoplasts in this case.

An anomaly in these experiments is that whereas the peak density of antibody binding to the plasmalemma as judged by topological labelling is 1.14 kg/l, the slot blot assay gives a peak density of 1.17 kg/l. Antibody reactivity at 1.17 kg/l in the labelling of intact protoplasts is roughly 80% of that at 1.14 kg/l, whilst at 1.14 kg/l the slot blot reactivity is ca. 75% of that at 1.17 kg/l. That these two peaks do not coincide may be the result of different requirements for immunodetection in the two assays, as outlined above. It has been noted that inside-out and right-side-out vesicles behave differently with regard to phase partitioning (Larsson *et al.*, 1984), isoelectric

focussing (Griffing and Quatrano, 1984) and lectin binding (Haass et al., 1985), whilst differences in density between different sided vesicles have been suggested to cause the non-coincidence of  $K^+$ -ATPase and GS-II enzyme activities occasionally observed in density gradients (Quail, 1979). From the results recorded here, it would be postulated that right-side-out plasmalemma vesicles have a higher density than inside-out vesicles. An antibody recognising an epitope expressed on the inside face of the plasmalemma would provide useful corroboration of such an hypothesis.

#### Comparison with classical plasmalemma markers.

Direct comparison with classical plasmalemma markers proved unsuccessful.  $Mg^{2+}$ -ATPase activity in gradients showed a large maximum at ca. 1.10 kg/l, and another at 1.17 kg/l, but little  $K^+$  stimulation at any density. There was insignificant inhibition by 50  $\mu$ M vanadate, or by oligomycin. In contrast, Beta vulgaris membranes showed a peak of vanadate sensitive  $K^+$ -ATPase activity at 1.18 kg/l. No peak of glucan synthase I or II activity was seen for N. glutinosa membranes (results not shown). PTAC staining was not attempted. Thus it appears that N. glutinosa suspension cultures are not an appropriate tissue on which to perform several of the classical marker assays. It is for such tissues that plasmalemma specific monoclonal antibodies will be a useful adjunct to previously available markers.

In view of these problems, any comparison with other markers must be indirect, relying on published reports. First, the peak density determined for the plasmalemma (1.14 kg/l) is rather low. Most published reports for a variety of species suggest that the plasmalemma has a density between 1.16 and 1.22 kg/l. However, many of these reports deal with roots, or other differentiated tissues, particularly from monocots. Reports of the density of the plasmalemma from dicot suspension culture protoplasts are in agreement with the density recorded here, particularly if not dependent on the classical markers. Thus Galbraith and Northcote (1977) found that membranes of Glycine max suspension culture protoplasts which were labelled with



diazotized sulphanilic acid had a density of 1.14 kg/l. Con A labelled membranes from carrot suspension culture protoplasts were also found to have a density of 1.14 kg/l in renografin gradients (Boss and Ruesink, 1979), although since the gradient medium was different, these results are not necessarily comparable (see Chapter 1).

Based on enzyme markers, it is found in general that the plasmalemma from dicot species is of a lower density than that from monocots (Robinson, 1985). Poole *et al.* (1984) found that vanadate sensitive ATPase from growing Beta vulgaris roots had a peak density of 1.165-1.175 kg/l, whilst in dormant tissues it decreased to 1.127 kg/l. Bowles and Kaus (1976) found that pH 6.0 ATPase from Phaseolus aureus hypocotyls showed a peak of activity from 1.12 to 1.15 kg/l, although GS-II activity peaked at 1.17 kg/l. Such an observation might be related to the difference in sidedness of vesicles, as postulated for the dichotomy between slot blot and topological labelling experiments recorded here. It has been suggested (Quail, 1979) that GS-II activity might be exhibited on right-side-out, and K<sup>+</sup>-ATPase activity on inside-out vesicles, due to the different topological requirements for substrate access to the active sites of the two enzymes.

That the 16.4B4 antigen appears to be expressed on intracellular membranes is perhaps not surprising. As discussed subsequently (Chapter 9) secretory and membrane bound glycoproteins synthesised on the endoplasmic reticulum pass through the Golgi to the plasmalemma. In the endoplasmic reticulum and/or Golgi, they acquire carbohydrate moieties, which undergo subsequent processing in the Golgi to the mature form. It would thus be expected that antibody labelling of membranes would detect antigen of heterogeneous density. Pulse-chase experiments using labelled amino acids or sugars would be a useful adjunct to these studies, since it might be possible to follow the maturation of the glycoprotein *en route* to the plasmalemma. Unfortunately, it did not prove possible to incorporate sufficient labelled amino acid into the 16.4B4 antigen to perform such experiments. Radiolabelling with sugars might prove more successful,

but was not attempted.

In Chapter 6, it was shown that a proportion of the 16.4B4 antigen was buffer soluble. This might correspond to the lower density peak of antibody binding seen in slot blots and on labelling of protoplast homogenates. That this peak has a variable density and occurs at the top of the gradient suggests that it might not be membrane bound. Since it is not present when intact protoplasts are labelled, this peak must be intracellular. Thus it would appear that *N. tabacum* cells contain an intracellular pool of 16.4B4 antigen, which is only weakly membrane bound, ie: has the characteristics of a peripheral membrane protein.

The evidence of detergent solubilisation suggests that the remainder of the 16.4B4 antigen is rather intimately associated with membranes, since it requires treatment with denaturing detergents to be effectively solubilised (Chapter 6). Either an intracellular membrane with which the 16.4B4 antigen is associated is readily disrupted by cold buffer, or there is a pool of the glycoprotein which is less intimately associated with membranes than the mature form. The latter postulate has implications for the method of plasmalemma association of the 16.4B4 antigen. Whereas many integral membrane proteins have hydrophobic domains which anchor them in the lipid bilayer (Capaldi, 1982), others are anchored by covalently bound lipid (Low *et al.*, 1986), which is attached in the Golgi cisternae (Schmidt and Schlesinger, 1980). Since oligosaccharide addition to the polypeptide can precede lipid attachment (Johnson and Spear, 1983), it is possible that the buffer soluble antigen could represent this species without bound lipid. There is at present no evidence of covalently attached lipid in the 16.4B4 antigen, but this represents an interesting hypothesis.

The results recorded in this chapter indicate that there is considerable heterogeneity in isopycnic density of membranes exhibiting the 16.4B4 antigen, but that by the choice of appropriate labelling conditions this may be reduced to the exclusive labelling of

plasmalemma bound antigen, which is resolved as a single peak of 1.14 kg/l in sucrose density gradients. These results provide a rational basis for the isolation and characterisation of the plasmalemma, independent of classical markers, and for design of more efficient plasmalemma isolation techniques. None of these subjects are addressed in the current work, but are at present under investigation (M.G. Hahn, unpublished results).

CHAPTER EIGHT.

DETECTION OF HYBRIDS FROM PROTOPLAST FUSION.

## INTRODUCTION.

The bulk of this thesis has been directed towards the increased characterisation of the structures recognised by the antibodies secreted by the hybridomas in the library. In contrast, this chapter deals with the potential use of such antibodies as selection tools in protoplast fusion experiments.

Somatic hybridisation by protoplast fusion is a potentially valuable method of genetic transfer between plants. Since sexual incompatibility barriers are circumvented (Gleba and Hoffmann, 1980; Krumbiegel and Scheider, 1981; Melchers, 1980), the range of traits which can be introduced is increased above that available from the sexual mating used in classical breeding methods. The lack of suitable markers to select and isolate the desired hybrids has, however, presented a problem in exploitation of this method. In contrast to many animal, fungal and bacterial species, where a large number of selectable genetic markers are available in a variety of cell types, for plants there is a paucity of such markers. Nevertheless, there have been reports of selection of heterokaryons by complementation of auxotrophic mutants or growth requirements (Maliga *et al.*, 1977; Harms *et al.*, 1981), or by enhanced division frequency of the hybrid protoplasts (Gleba and Hoffmann, 1978). Others have used morphological markers, such as colour differences between fused and unfused protoplasts (Potrykus, 1972; Flick and Evans, 1983), and inclusion of both chloroplasts and starch granules (Kao *et al.*, 1974; Constabel *et al.*, 1976) to manually select heterokaryons. Recently, there have been reports of the uptake of fluorochromes by protoplasts or cells prior to protoplasting, and their use as markers in cell fusion experiments (Galbraith and Galbraith, 1979; Galbraith and Mauch, 1980; Kanchanapoom *et al.*, 1985; Alexander *et al.*, 1985). Although free fluorochromes have potential as fusion markers, their uptake into cells is variable, and may have unknown effects on cell viability. In addition, fluorochrome uptake is slow, typically taking 15-18 h (Galbraith, 1984; Kanchanapoom *et al.*, 1985; Afonso *et al.*, 1985). Galbraith and Mauch (1980) suggested that fluorescein isothiocyanate

became covalently bound to cytosolic components upon uptake by protoplasts, but recorded no effect of this on their viability. It has been recorded that exposure of leach neurons to near ultraviolet light after their microinjection with fluorescein isothiocyanate-dextran conjugates results in selective cell death (Shankland, 1984). It is possible that irradiation of fluorophors could have a detrimental effect on plant cell viability, although none has been recorded.

Thus the use of monoclonal antibodies as markers in cell fusion experiments could have unique advantages over other currently available methods, being generally applicable and non-invasive. In this chapter, a number of experiments are described which suggest the potential utility of monoclonal antibodies as fusion markers. Fused protoplasts are definitively identified as a small percentage of the population. Although their isolation from the unfused population was not attempted, methods are discussed which would allow the rapid and efficient selection of large numbers of hybrid protoplasts on the basis of surface antibody fluorescence.

## MATERIALS AND METHODS.

### Preparation and labelling of protoplasts.

Protoplasts were prepared from *N. glutinosa* suspension cultures, leaves from *N. tabacum* plants grown *in vitro*, and leaves from greenhouse grown *P. vulgaris* plants as described in Chapter 2. Where indicated, they were labelled with monoclonal antibodies in sorbitol osmoticum as described in Chapter 4. 16.4B4 antibody was from ascites fluid diluted 1:250 in sorbitol osmoticum (0.4 M sorbitol, 0.2% HEPES, pH 7.2 containing 1 mg/ml BSA and 0.1 mg/ml goat gammaglobulins). 17.3D4 antibody was from culture supernatant diluted 1:10 in the same solution. Rhodamine and fluorescein conjugated goat anti-mouse antibodies (HyClone, Logan, Utah) were used at 1:10 dilution in the above buffer.

### Protoplast fusion.

Protoplast fusion was by a modification of the method described by Evans (1983).  $2 \times 10^5$  protoplasts of each parental type were mixed in a 17 x 100 mm Falcon (Oxnard, California) tube, and centrifuged at 100 g for 2 min. The supernatant was aspirated to 300  $\mu$ l, and the protoplasts gently resuspended. To the suspension was added 900  $\mu$ l of a 25% (w/v) solution of polyethyleneglycol 1500 (BDH, Poole, Dorset; batch 5961400C) in 200 mM glucose, 700 mM  $\text{KH}_2\text{PO}_4$ , 10 mM  $\text{CaCl}_2$ , pH 5.8. Following incubation at room temperature for 20 min, the suspension was diluted at 10 min intervals with two 1.8 ml aliquots of 300 mM glucose, 50 mM  $\text{CaCl}_2$ , 50 mM glycine, pH 10.5. 10 min after the second addition, the supernatant was removed from the protoplasts which had settled on the bottom of the tube, which were diluted with a 1 ml aliquot of regeneration medium. A second 1 ml aliquot of regeneration medium was added after a further 10 min. An aliquot from the fused protoplasts was removed for observation by epifluorescence microscopy. The Nikon Diaphot inverted microscope was fitted with a B filter block (excitation wavelength 460-485 nm, barrier filter 515 nm) and a G filter block (excitation wavelength 535-550 nm, barrier filter 580

nm). Photography used Kodak Ektachrome ASA 400 colour film.

#### Culture of labelled protoplasts.

Antibody labelled protoplasts were cultured at  $2 \times 10^4$  to  $1 \times 10^5$ /ml in a regeneration medium comprising 2% (v/v) coconut milk, 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 1.0 mg/l kinetin, 200 mg/l casein hydrolysate, 2% sucrose and 0.2 M each of mannitol and sorbitol with the organic acids, vitamins, sugars and sugar alcohols of Kao (1977), at pH 5.7. Culture was in 60 mm petri dishes, initially in the dark for 24 h, thereafter on a 14 h light: 10 h dark cycle at 28°C. 1-2 ml of fresh medium was added weekly, the first addition being at a (sorbitol + mannitol) concentration of 0.3 M, subsequent additions being at 0.2 M. After 1 month, the cells were transferred to suspension culture, and grown in the dark at 28°C in 100 ml of MS medium containing 0.5 mg/l 6-benzylaminopurine and 0.5 mg/l 2,4-dichlorophenoxyacetic acid in 250 ml conical flasks on a rotary shaker at 100 rpm.

After 1-2 weeks of growth in culture, cells were transferred to 100 ml of MS medium containing 1.0 mg/l 6-benzylaminopurine, 0.2 mg/l indoleacetic acid and 0.8% agar in GA 7 culture vessels (Magenta Corporation, Chicago, Illinois). Following maintenance on a 14:10 dark:light cycle for 2 weeks, shoots which had formed were transferred to GA 7 vessels containing MS medium without hormones. Rooting ensued, and plantlets were transferred to soil and grown as for *P. vulgaris* plants (see Chapter 2).



## RESULTS.

### Stability of label during cell fusion.

That the binding to protoplasts of fluorescently labelled antibodies was stable to the conditions necessary to effect cell fusion was demonstrated by the fusion of labelled *N. tabacum* leaf protoplasts with unlabelled *N. glutinosa* suspension culture protoplasts. Following fusion, fluorescent antibody was observed to be confined almost exclusively to protoplasts containing chlorophyll, as judged by the red chlorophyll fluorescence seen using the B filter block. A small number of leaf derived protoplasts contained no chloroplasts, and showed only green peripheral fluorescence from the antibody label. They were, however, distinguishable morphologically from suspension culture derived protoplasts, as they were smaller in size and contained less pronounced nuclei and cytoplasmic strands. By counting fields of protoplasts, it was determined that the number of such protoplasts before and after fusion was equivalent. Similarly, no loss or transfer of label was seen in mixed but unfused protoplast populations.

### Fusion of labelled suspension culture protoplasts with leaf protoplasts.

Antibody labelled *N. glutinosa* suspension culture protoplasts were fused with unlabelled *N. tabacum* leaf protoplasts. In this case, surface antibody-linked fluorescence was used as a first marker, and chlorophyll autofluorescence as a second to detect hybrids. Fig. 8.1 a and b show the *N. glutinosa* parent, and c and d the *N. tabacum* parent before fusion. Fig. 8.1 e and f show an unfused protoplast of each parental type prior to the fusion.

After fusion, about 4% of protoplasts showed both chlorophyll autofluorescence and antibody linked surface fluorescence (Fig. 8.1 g and h), indicating that they were heterokaryons formed by interspecific fusion. No green fluorescence was observable transecting

the fused protoplasts, indicating that they had indeed fused. Unfused aggregates clearly showed intervening plasmalemma (result not shown).

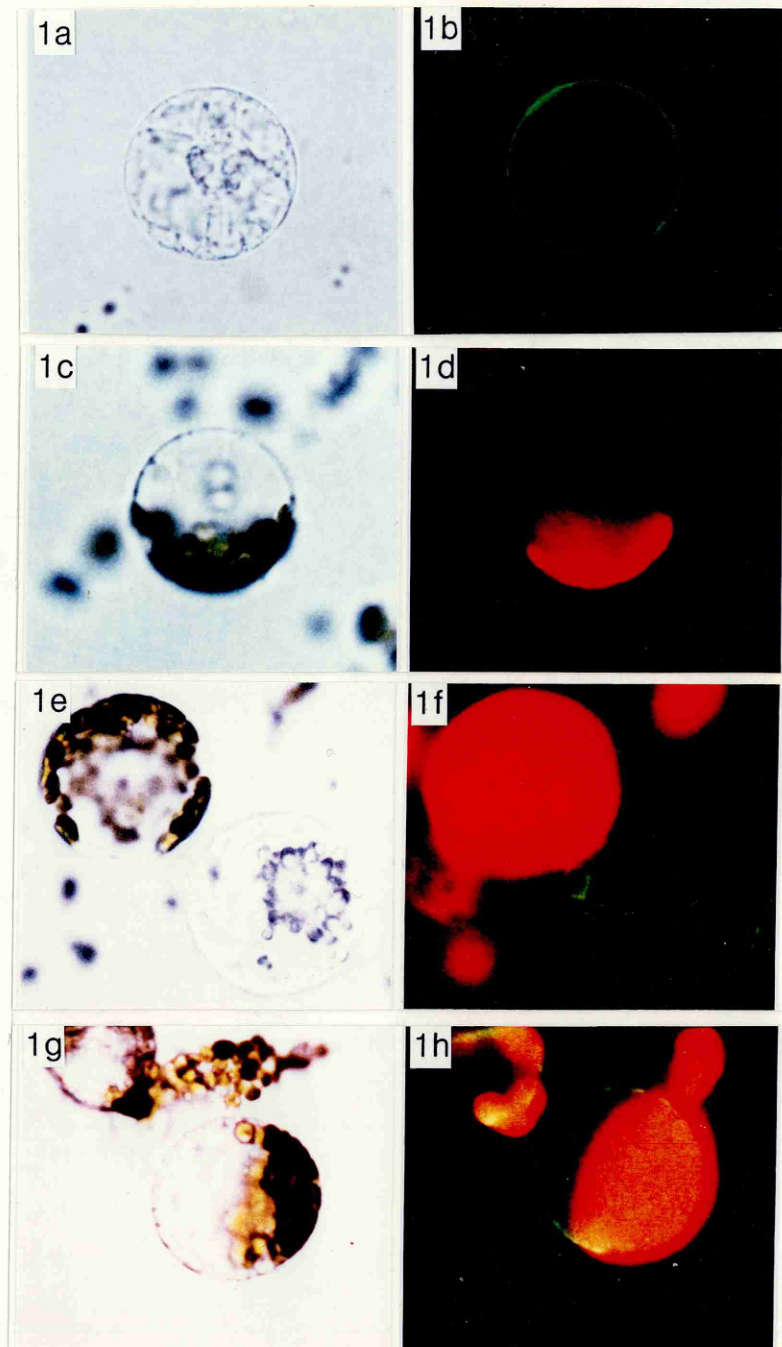


Figure 8.1. Fusion of *N. glutinosa* suspension culture and *N. tabacum* leaf protoplasts.

Fluorescence labelled *N. glutinosa* suspension culture protoplasts were fused with unlabelled *N. tabacum* leaf protoplasts. a, c, e and g: brightfield micrographs of *N. glutinosa* (a) and *N. tabacum* (b) parents, one of each parent in a mixture (e) and a heterokaryon formed by fusion (g). b, d, f and h: fluorescence micrographs of the corresponding protoplasts.

## Fusion of two antibody labelled populations.

The antibody linked fluorescence based selection procedure may be made entirely general by fusion of two populations of antibody labelled protoplasts. In this case, different populations of protoplasts are labelled with monoclonal antibody followed by different second antibodies, one rhodamine- and one fluorescein-conjugated. Hybrids were detected by the presence of both labels on the plasmalemma. To demonstrate the procedure, fusion was effected between two lots of *N. glutinosa* protoplasts. Both were labelled with 16.4B4 monoclonal antibody, for one lot followed by rhodamine conjugated goat anti-mouse antibody, whilst the other lot was labelled with fluorescein conjugated second antibody.

After fusion most protoplasts showed either red or green surface fluorescence. From 3-5% of protoplasts showed hemispheres of red and green fluorescence. Fig. 8.2 b and c show the two halves of a single protoplast (Fig. 8.2 a). Fig. 8.2 d is a double exposure photograph showing another fused protoplast, using both excitation filters. There was little evidence of substantial mixing of the hemispheres of fluorescence, even several hours after fusion.

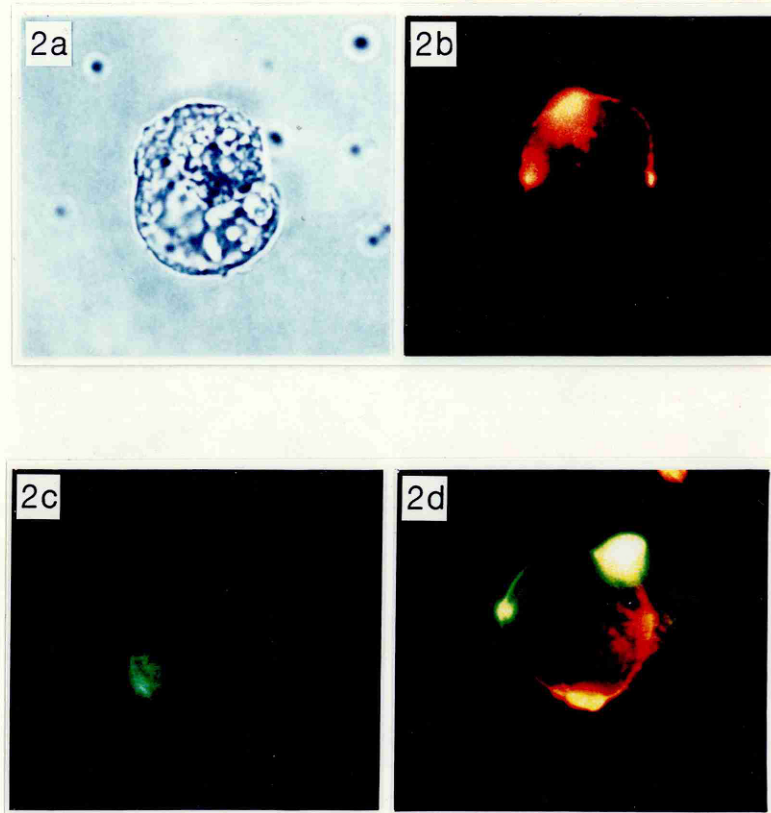


Figure 8.2. Fusion of rhodamine and fluorescein labelled N. glutinosa protoplasts.

N. glutinosa protoplasts were labelled with 16.4B4 antibody, then rhodamine or fluorescein conjugated goat anti-mouse antibodies. (a) brightfield (b) rhodamine and (c) fluorescein fluorescence micrograph of a single hybrid. (d) rhodamine/fluorescein fluorescence double exposure of another fusant.

Selective labelling following fusion.

Perhaps the most significant advantage of monoclonal antibodies over other markers in fusion experiments is the potential of appropriate antibodies to select subpopulations of protoplasts from the parental contingent. Although none of the antibodies available selectively bind to subpopulations of protoplasts, the potential of the method may be demonstrated using hybridoma supernatant 17.3D4.

A series of dilutions of 17.3D4 in sorbitol osmoticum were incubated separately with equal numbers of P. vulgaris leaf and N. glutinosa suspension culture protoplasts. At high concentrations of supernatant such as 1:1 and 1:2 dilutions, fluorescence was equally bright on the surface of protoplasts of both species. With greater supernatant dilution, however, antibody binding to the P. vulgaris protoplasts decreased more rapidly than that to N. glutinosa protoplasts. It was found that at a 1:10 dilution of supernatant in sorbitol osmoticum, antibody binding was almost exclusively to N. glutinosa protoplasts in a mixed population, as judged by the small number of chlorophyll containing protoplasts showing antibody linked surface fluorescence (Fig. 8.3 a and b). Following fusion of mixed unlabelled N. glutinosa and P. vulgaris protoplasts, these were labelled with 17.3D4 supernatant diluted 1:10 in sorbitol osmoticum, followed by fluorescein conjugated goat anti-mouse antibody. About 4% of the protoplasts were heterokaryons, as judged by the internal P. vulgaris derived chloroplast fluorescence, and surface N. glutinosa epitope dependent fluorescence (Fig. 8.3 c and d).

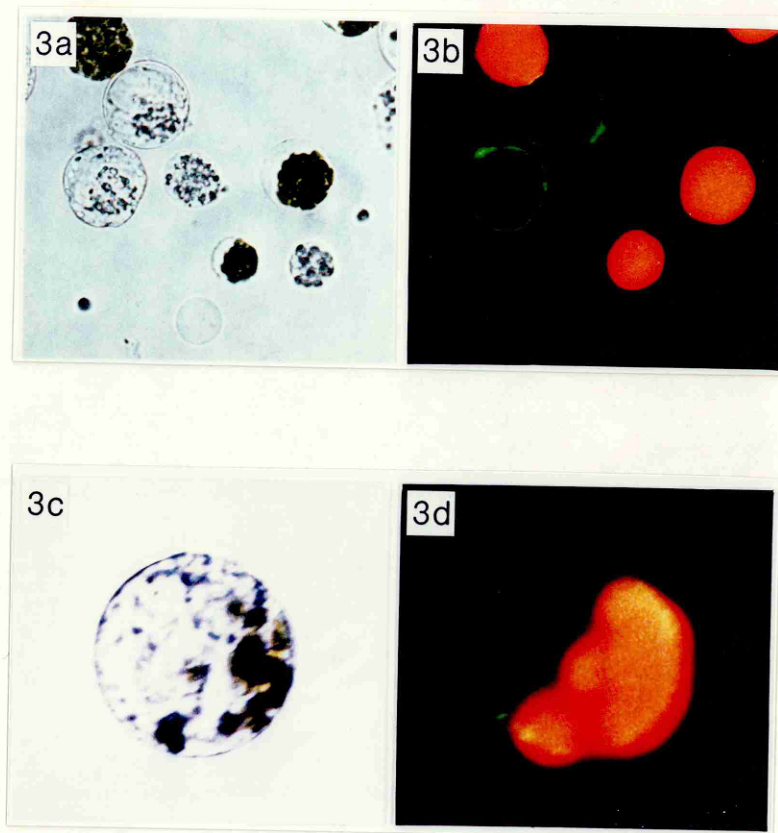


Figure 8.3. Antibody labelling following fusion of N. glutinosa suspension culture and P. vulgaris leaf protoplasts.

A fused population of N. glutinosa suspension culture and P. vulgaris leaf protoplasts was labelled with 17.3D4 hybridoma supernatant diluted 1:10 in IF wash, followed by fluorescein labelled goat anti-mouse antibody. (a) and (c): brightfield micrographs and (b) and (d): fluorescence micrographs of (a and b): a mixed population of protoplasts, and (c and d): an interspecific fusant.

## Regeneration of labelled protoplasts.

For these procedures to be useful, it is important that the labelled and fused protoplasts be able regenerate into cell cultures and plants. Although the protoplasts were not selected after fusion, this property may be demonstrated on the labelled but unselected cultures. N. tabacum protoplasts were labelled with monoclonal antibody 16.4B4 and fluorescein conjugated goat anti-mouse antibody. When viewed by fluorescence microscopy, all the protoplasts in the population were seen to be labelled. These protoplasts reformed cell walls when cultured in regeneration medium, underwent cell division and formed a morphogenetically competent suspension culture. Plantlets could be regenerated from this culture, which could be grown to maturity under greenhouse conditions. There was no difference in the efficiency of any of these processes between labelled and unlabelled control protoplasts. Similarly, plantlets could be regenerated after subjecting a mixture of N. glutinosa and N. tabacum protoplasts to the fusion procedure.



## DISCUSSION.

This chapter describes the results of experiments designed to demonstrate the potential usefulness of monoclonal antibodies as markers in cell fusions. Appropriate combinations of antibody and protoplast afford considerable versatility to this method of cell selection, from the most general applicability to high selectivity for specific fusants.

The fusion of suspension culture and leaf mesophyll derived protoplasts has been used previously as a basis for the selection of hybrids, since the two parental types are morphologically distinguishable (Kao *et al.*, 1974; Constabel *et al.*, 1976). The presence of chloroplasts provides an intrinsic marker for the leaf derived parent in a fusion. The use of monoclonal antibody linked fluorescence as an additional marker for the suspension culture derived parent is a useful addition to this protocol, since it provides a positive method of identification of the second parental type. As predicted, fusants have both surface green and internal red fluorescence when viewed under appropriate epifluorescence illumination. Further, the proportion of fusants (4%) is in the range expected for a successful fusion experiment, strengthening the conclusion that only true fusants are being detected.

The fusion of protoplasts labelled with rhodamine and fluorescein conjugated antibodies demonstrates the additional flexibility of monoclonal antibody labelling over morphologically based selection. Thus, populations of morphologically, phenotypically and genetically identical protoplasts may be fused, and hybrids selected on the basis of their dual antibody labelling. Although the example of fusion of identical *N. glutinosa* protoplasts used to demonstrate the method probably has little utility beyond the selection of polyploids, it is a considerable advantage to be able to select heterokaryons from fusion of protoplasts derived from any plant part, with any genetic background. The method would be equally applicable to selection of fusants of two leaf derived protoplast populations. Thus, using

surface selective antibodies of suitably general specificity, it is possible to use antibody based detection as a completely general selection tool.

Antibody based detection can also be tailored to select fusants between specific sets of protoplasts based on the inherent selectivity of appropriate monoclonal antibodies. Although none of the antibodies generated in the current study show much specificity of reactivity (see Chapter 3), probably for the reasons outlined in Chapter 2, certain antibodies (eg: 16.4B4, 17.3D4) show family selectivity. Of these, 17.4D4 was found to show the greatest selectivity between P. vulgaris and N. glutinosa protoplasts, although this was not seen until antibody concentration was titrated to an appropriate dilution. Above this level, there was cross-reactivity with the P. vulgaris protoplast plasmalemma. Thus the antibody, at appropriate dilution, could be used to detect heterokaryons after fusion had been effected. The similarity of the level of fusion detected to that in previously described experiments suggests that antibody labelling post-fusion is not detecting surface antibody fluorescence on an appreciable number of non-fused P. vulgaris protoplasts. The selection of specific protoplasts from the population based on antibody binding has considerable potential, when appropriate monoclonal antibodies are available.

As has been discussed (Chapter 1), different subpopulations of mammalian cells express unique monoclonal antibody defined surface epitopes, which provide the basis of positive and negative selection of such cells. It is plausible that appropriate immunisation and screening strategies will unearth such surface epitope defined subpopulations of plant cells. It might, for example, be possible to select meristem derived protoplasts from populations. These might show increased potential for regeneration, and confer such potential on hybrids with non-regenerant protoplasts. If this were the case, post-fusion antibody selection might allow the regeneration to plants of hybrids of recalcitrant species, amongst which are many important crop plants. Such hybrids would have a novel genetic constitution.

## General considerations.

The term "selection" has been used at some points in this discussion. Although the experiments described here do not include selection of hybrids from the population, recent advances in the application of fluorescence activated cell sorting to plant protoplasts afford the potential to rapidly select large numbers of interspecifically fused protoplasts from a population.

Afonso et al. (1985) have described the selection of protoplast heterokaryons by fluorescence activated cell sorting. Parental protoplasts were labelled by uptake of FITC or RITC from culture media, and fusants selected on the basis of the presence of both fluorochromes in a single particle. In addition, hybrids of FITC labelled suspension culture and unlabelled leaf protoplasts were selected by their dual fluorescence. Selected protoplasts were regenerated to plants, the hybrid nature of which was proved by isozyme analyses. After sorting, the protoplast populations contained 37-90% heterokaryons, an enrichment of 33- to 390-fold over the unsorted population.

Jett and Alexander (1985) undertook an extensive investigation of instrument parameters necessary for efficient sorting of plant protoplasts, modifying a number of conditions from those used previously to sort protoplasts. Alexander et al. (1985) used the modified instrument to select heterokaryons from a fused population, resulting in a 225-fold enrichment in heterokaryons from 0.2 to 45% of the protoplast population. Regeneration of plants was not attempted. Contamination of the sorted heterokaryons was largely with non-fused aggregated protoplasts. Large aggregates were removed by sieving prior to sorting, and aggregates which were initially selected by the machine could be removed by resorting the selected protoplasts (P.J. Jackson, personal communication).

Detection of heterokaryons using antibody linked fluorescence lends itself to large scale selection using flow cytometry, both since

instruments have been designed to select antibody labelled animal cells (Redenbaugh et al., 1982), and because surface labelling provides a favourable geometry for detection (P.J. Jackson, personal communication). In addition, use of an electronic doublet discriminator during sorting should allow the differentiation of aggregates from true heterokaryons (J.H. Jett, personal communication).

Of the heterokaryons which are generated in a protoplast fusion experiment, many will retain separate nuclei. A proportion of the fused cells will undergo nuclear fusion, resulting in formation of a synkaryon. Such cells have a tendency to lose chromosomes, becoming aneuploid and losing the ability to express the full genetic complement of both parents (Kao, 1977; Dudits et al., 1980; Hoffmann and Adachi, 1981). A genetically based selection process results in the selection only of those hybrid cells in which genetic deficiencies of the parents are truly complemented. This represents a more limited selection criterion than one based on physical fusion of the plasmalemma, so that protoplasts selected on the latter basis have the potential to show more genetic variation. This may well be an advantage in the use of protoplast fusion to select phenotypically variant hybrid plants, since there is a greater potential to derive plants of novel genetic constitution. For example, Evans et al. (1982) reported the fusion of protoplasts from a homozygous albino suspension culture of *N. tabacum* with leaf protoplasts of *N. glauca*. Hybrid plants were recognised on the basis of light green leaf phenotype. Afonso et al. (1985), using fluorescence activated cell sorting, selected a wider variety of heterokaryons from the fusion between protoplasts of these two species, which showed greater genetic variability.

Refinements in the specificity of monoclonal antibodies used, along with fluorescence activated sorting of heterokaryons, followed by regeneration of plants and field testing might provide the basis of a novel and powerful method of generating hybrid plants with useful new characteristics.

CHAPTER NINE.

GENERAL DISCUSSION.

Number and localisation of antigens.

The work recorded here has generated and defined a number of novel antigenic markers within the plant cell. Starting with a crude cellular homogenate, it has proved possible to derive hybridoma lines secreting monoclonal antibodies reactive with different sites in the plant cell. Whereas a set of antibodies defines the exterior of the cell wall, a second non-overlapping set reacts with the plasmalemma. Yet a third set appears to recognise intracellular epitopes. There is considerable evidence that more than three epitope specificities are represented by these groups. The cell wall reactive group consists of the antibodies secreted by hybridomas 17.3B4 and 23.2D3. Whereas both these specificities were exhibited in the suspension cultures used in the initial immunofluorescence microscopy screening, both were lost after further subculture. 17.3B4 but not 23.2D3 reactivity could be regained by culture of the cells at higher density, indicating that the epitopes recognised differ. The former was also slightly reactive on Western blots, whereas the latter was not.

Western blot analyses of the plasmalemma reactive clones (Chapter 5) suggested that at least three different antigens were recognised. 16.2C6, 16.4A6 and 16.4B4 recognised a common 130-230 kd band, whilst 16.1B3 and 16.3C1 had a different pattern of reactivity. 17.3D4 and 17.4B5 showed no reactivity on the Western blots. Clones in the other libraries showed at least four other specificities for plasmalemma expressed epitopes.

By Western blot analysis, other hybridoma libraries showed a variety of different reactivities against internal epitopes, but none were detectable by this method for the *N. glutinosa* library. Two different anti-protein antibody specificities from this library were, however, detectable by immunoprecipitation of *in vivo* radiolabelled proteins. In contrast to the plasmalemma reactive antibodies, most of which showed diffuse staining of electrophoretically separated proteins on Western blots, many internally expressed antigens were of a unique molecular weight. This observation will be discussed in

greater detail subsequently.

A major obstacle to the interpretation of the data is that the unique plasmalemma localisation of the epitopes recognised on the surface of intact protoplasts has not been defined. The results of the slot blot and topological labelling studies on density gradient resolved membranes suggest that such epitopes are also expressed on several organelles within the cell. It is indeed very likely that plasmalemma expressed epitopes are assembled on other membranes within the cell and pass through intracellular organelles en route to the plasmalemma. This possibility will also be discussed further subsequently.

If the antigens are indeed in transit between different organelles, how can we define that the plasmalemma is the terminal organelle of association, rather than that the antigens are secreted into the cell wall, or reabsorbed into the intracellular space? The latter is quite likely, since there is evidence in plants (Tanchak *et al.*, 1984) for the recirculation of membrane constituents extensively documented in animal cells (Pearse and Bretscher, 1981; Pastan and Willingham, 1981).

Such questions are best answered by ultrastructural studies of plant cells. Immuno-electron microscopy provides a powerful tool to define the subcellular sites of expression of the recognised epitopes. For example, Brewin *et al.* (1985) were able to show using immuno-gold labelling that a monoclonal antibody raised against the peribacteroid membrane in pea also reacted with the plasmalemma and Golgi. Such studies would be useful in establishing the sites of recognition of the plasmalemma reactive antibodies, and the organelles involved in processing the recognised epitopes, and might also illuminate the events and selectivity involved in endocytosis in plant cells. Immuno-electron microscopy would also be useful in defining the distribution of intracellular antigens, some of which might provide novel markers for important intracellular organelles. There is, for example, a paucity of markers for the tonoplast (Wagner, 1983), and

nuclear envelope (Dunham and Bryant, 1983), both of which are of biological interest.

#### Antigenicity and immunodominance.

It is interesting that the mammalian cell fusions produced a high proportion (7 of 14) of hybridomas which secreted antibodies to plasmalemma expressed antigens. Of the hybridomas, all five originating from mouse 16 were plasmalemma specific, and showed but two specificities. In contrast, hybridomas derived from mouse 17 were of wider specificity. Only two reacted with the plasmalemma, these apparently being of different specificities from each other, and from the mouse 16 hybridomas. Of the other mouse 17 hybridomas, one reacted with the cell wall, whilst three were directed against internal epitopes. Mouse 23 produced one hybridoma line secreting antibodies against the cell wall and one against an internal epitope. Although this does not represent a statistically significant sample, it would appear that there were considerable differences in the immune responses of the different mice; this is particularly striking in the case of mice 16 and 17, which were siblings of an inbred mouse strain, subjected to the same immunisation schedule using the same immunogen preparation, and sacrificed within three hours of each other. A similar phenomenon may be seen in the reactivities of polyclonal antisera derived from mice 25, 26 and 27 (Fig. 6.3), which also show differences in reactivity pattern for three inbred siblings treated identically.

On the other hand, three different investigators using Balb/c mice from different litters, different immunogen preparations from different Nicotiana species and different immunisation regimes generated five antibodies of identical specificity, 17.3A6 being the archetype. These observations are perhaps best explained on the basis of two properties of the mammalian immune system, namely the processes involved in generation of antibody diversity, and the structural characteristics of immunodominant epitopes, both of which have been the subject of intensive recent research.



Most estimates place the number of different specificities which may be generated by the mammalian immune system in the range  $10^6$ - $10^8$  (eg: Sigal and Klinman, 1978). On a molecular level, this diversity is expressed as differences in antibody combining sites, which are composed of six hypervariable regions of the immunoglobulin amino acid sequence. There are three basic mechanisms by which these differences are produced (reviewed by Tonegawa, 1983; Honjo, 1983).

First, there are multiple copies of the different gene segments which combine to produce the hypervariable region. One of 100-300 copies of the  $V_H$  and  $V_L$  is spliced to one of a number of J segments, which in the heavy chain is in turn joined to one of a number of D segments. Random combination of these gene segments during maturation of a B cell line is one source of antibody diversity. In addition, diversity is increased by heterogeneity in combining points between segments, whilst non-genome-encoded nucleotides can be added de novo between the gene segments during rearrangement in B cell maturation (Alt and Baltimore, 1982). Somatic mutations of functional genes in B cell lineages add yet more diversity.

Of these diversity generating methods, all but the use of multiple gene segments are somatic processes, and so are independent of genetic factors. Thus genetically inbred littermates have the potential to elaborate different immune repertoires. Similar epigenetic mechanisms determine the specificity of T cell clones which regulate the B cell immune response (Hedrick et al., 1984). It is conceivable that differences in somatic recombination events occurring in different mice could create differences in the manner in which they react to an identical stimulus. Such processes might explain the differences in immune response to the same set of proteins seen in the initial fusion experiments, or to a purified antigen as reported in Chapter 6.

The independent production of a number of hybridomas secreting antibodies reactive against the same epitope suggests that the epitope is immunodominant in the immunogen preparation. In any antigen preparation, whether a mixture of substances or a pure protein, a

small number of epitopes are found to elicit a disproportionately strong immune response. In the case of pure proteins, the factors determining immunodominance are being dissected.

The immunodominant epitopes of the influenza virus haemagglutinin have been mapped (Laver *et al.*, 1980; Webster and Laver, 1980). These are restricted to a small area of the molecular surface (Wiley *et al.*, 1981), whereas by immunisation with synthetic peptides corresponding to parts of the molecule, antibodies reactive with many other parts of the structure may be raised (Green *et al.*, 1982).

In similar mapping studies by Westhof *et al.* (1984), on the antigenic determinants on tobacco mosaic virus protein, myoglobin, and lysozyme, antigenicity was compared to the atomic temperature factors—the mean square displacement of atoms in the folded protein as derived from X-ray crystallography studies. It was found that antigenicity resides in amino acid sequences having high temperature factors. The same conclusion was reached by Tainer *et al.* (1984) from studies of the antigenicity of myohaemerythrin, and synthetic peptides homologous to parts of its sequence. Thus it appears that antigenic regions of proteins possess high segmental mobility.

This result has been interpreted in terms of an induced fit model of antigen:antibody interaction (Westhof *et al.*, 1984; Tainer *et al.*, 1984; Tainer *et al.*, 1985). Unlike haptens, the binding of which is confined to a small, rigidly defined pocket in the complementarity determining region (Amzel *et al.*, 1974; Segal *et al.*, 1974), with protein antigens it is envisaged that initial interaction of the antigen with the antibody is followed by segmental mobility of the antigenic region to maximise the number of intermolecular contacts and increase the binding energy (Williams and Moore, 1985). Rees and de la Paz (1986) have suggested that chain length variations in the complementarity determining regions increase the number of surface topologies of the combining sites, and act with amino acid sequence variation of the combining site to increase antibody diversity. Computer modelling of the interaction of hen egg white lysozyme with

an antibody (Gloop 2; Darsley and Rees, 1985) suggests that antigen contact may occur over a large surface of roughly 4 x 5 nm, including contributions from all of the antibody's complementarity determining regions. X-ray diffraction studies of antibody-lysozyme complexes are in agreement (Amit *et al.*, 1985).

The carbohydrate moieties of glycoproteins are likely to be the most mobile component of their structure. Whereas the protein core will have a defined conformation, in order to fulfil its function, the attached oligosaccharides have considerable freedom of motion around the bonds between residues, unless this is constrained sterically or by hydrogen bonding. Thus they are likely to be more immunogenic than the more ordered core protein, for the reasons discussed above. A counterpoint to this argument is the limited range of patterns of glycosylation exhibited by many glycoproteins (see below). If an oligosaccharide moiety of a glycoprotein does not contain carbohydrate residues or linkages foreign to the host, it is likely that the animal will be tolerant to the carbohydrate moiety of an immunogen, and so will not mount an immune response against it. Thus the carbohydrate moieties of glycoproteins are usually considered to be weakly immunogenic. This is probably not an intrinsic property of such groups, however, since oligosaccharides of composition unusual to animals are highly immunogenic, as evidenced by the studies of Gleeson and Clarke (1980) and Anderson *et al.* (1984). In addition, the sugar residues will be highly solvated, and in a highly glycosylated protein such as the 16.4B4 antigen may sterically hinder the access of large molecules such as antibodies to the core protein (see below). A single core protein might also carry a number of similar oligosaccharides, increasing the likelihood that an oligosaccharide determinant would be recognised. Thus it is perhaps not surprising that monoclonal antibody 16.4B4 recognises a carbohydrate epitope on the antigen.

It also seems that much of the reactivity of the polyclonal antisera described in Chapter 6 is against such epitopes, despite the expected increase in accessibility of the core protein caused by its denaturation and removal from the membrane prior to immunoaffinity

chromatography. There does, however, seem to be a periodate insensitive component to the reactivity of the antiserum, which is to a band in Western blots of the same molecular weight as the deglycosylated 16.4B4 antigen. Thus it seems possible that the polyclonal antiserum contains components that are reactive against carbohydrate epitopes other than those recognised by 16.4B4, and also reactivity against the core protein. A similar range of reactivities is seen in the monoclonal antibodies raised against the purified antigen.

In the case of the 17.3A6 antigen, it seems likely that the immunodominant epitope is proteinaceous. The 17.3A6 antigen appears to be synthesised at a high rate in suspension culture cells, and may also be a major intracellular protein (Chapter 5). Thus its high antigenicity may be the result both of its representation at high levels in the antigen preparation, and possibility of high segmental mobility of the immunodominant epitope.

#### Glycosylation of plant proteins.

The microheterogeneity evident as diffuse banding on Western blots following polyacrylamide gel electrophoresis (Chapter 5), coupled with the periodate oxidation studies (Chapter 6), suggest that many of the protein antigens which are recognised by the monoclonal antibodies of the library are glycosylated. Thus it is perhaps useful to review the evidence for and mechanisms involved in glycosylation of plant proteins.

In plants, the amino acid-sugar linkages found are beta-D-N-acetylglucosaminylasparagine, alpha-D-galactosylserine, alpha-D-xylosylthreonine, beta-D-galactosylhydroxyproline, beta-L-arabinosylhydroxyproline and possibly beta-L-glucosylhydroxyproline (Lamport, 1980). Of these, the last three are unique to plants. Hydroxyproline occurs in animal extracellular glycoproteins, such as collagen, but is not glycosylated.

The mechanism of N-glycosylation has been extensively investigated in yeast and mammals; the evidence suggests that similar mechanisms pertain in plants (Elbein, 1979). The processes involved in N-glycosylation, and the sites of reactions have been reviewed by Kornfeld and Kornfeld (1985) and by Dunphy and Rothman (1985). Initial N-glycosylation occurs cotranslationally in the rough endoplasmic reticulum (Katz et al., 1977; Bergman and Kuehl, 1978) from an oligosaccharide chain assembled on a lipid carrier composed of dolichol phosphate. All proteins receive the same core oligosaccharide of composition  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  (reviewed by Hubbard and Ivatt, 1981). In the rough endoplasmic reticulum, these chains are processed by removal of three glucose and one mannose unit, before export to the Golgi which occurs via transport vesicles (Jamieson and Palade, 1968).

In mammalian cell Golgi, a series of spatially segregated processing events converts the core oligosaccharide to one of four structures. In complex type oligosaccharides, additional N-acetylglucosamine, galactose and sialic acid residues are added, plus in some cases fucose and uronic acids. In high mannose structures, mannose residues are added up to a three branched  $\text{Man}_9\text{GlcNAc}_2$  structure. Hybrid oligosaccharides have characteristics of both types (Kornfeld and Kornfeld, 1985). Lysosomal enzymes undergo phosphorylation of outer mannose residues in the cis Golgi cisternae, which appears to be the signal targetting them to the lysosome (Reitman and Kornfeld, 1981; Varki and Kornfeld, 1981). The controls of the pathway of oligosaccharide processing taken by a particular glycoprotein are unknown, but may involve secondary structure around the core oligosaccharide (Hunt et al., 1983; Rosner et al., 1980; Green, 1982). The signal for core N-glycosylation appears to be the sequon Asn-X-Thr/Ser where X can be any amino acid except proline. Lehle and Bause (1984) examined the efficiency of glycosylation of synthetic peptides by a membrane extract from Saccharomyces cerevisiae. They found that the minimum requirement for core glycosylation was a tripeptide as above, with blocked asparagine alpha-amino and hydroxy amino acid alpha-carboxyl groups, but that the efficiency of glycosylation increased with increasing polypeptide

length. However, only about one third of Asn-X-Ser/Thr sequences in proteins are actually glycosylated (Kronquist and Lennarz, 1978).

Dolichol phosphate linked  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  is transferred to protein in plant cells (Lehle, 1981; Dorland *et al.*, 1981). As in mammalian and yeast cells, dolichol oligosaccharide synthesis is sensitive to the antibiotic tunicamycin (James and Elbein, 1980; Hori *et al.*, 1982). Processing to high mannose but not to complex or hybrid structures has been found in plants (Lehle and Tanner, 1983). There is no evidence of phosphorylation of oligosaccharides in plant cells. Vitale and Chrispeels (1984) found that processing and transport to the protein bodies (compartmentally analogous to lysosomes) of phytohaemagglutinin in developing *Phaseolus vulgaris* cotyledons involved modification of mannose content and addition of fucose, xylose and terminal N-acetylglucosamine to oligosaccharides, but no phosphorylation. The mature protein in the protein bodies contains no terminal N-acetylglucosamine residues, which presumably are removed at this site. The N-glycosylation sequon appears to be the same in plants as in other organisms (Sharon and Lis, 1979). Thus mechanisms of N-glycosylation appear to be similar in plants, yeast and mammals, although plants lack some of the modification pathways found in other species.

In contrast, plants can elaborate extensive O-linked oligosaccharides, many of which are secreted into the cell wall and middle lamella of plants or the medium of suspension culture cells. Hydroxyproline-rich glycoproteins of the cell wall contain numerous  $\text{Ara}_{1-4}\text{-O-Hyp}$  oligosaccharides, as well as  $\text{Gal-O-Ser}$  linkages (Lamport, 1967; Lamport *et al.*, 1973).  $\text{Gal-O-Hyp}$  is found in arabinogalactan proteins and algal cell walls, but not hydroxyproline-rich glycoproteins (Fincher *et al.*, 1974).

Unlike the signals for and steps involved in N-glycosylation, O-glycosylation is poorly understood. Lehle *et al.* (1977) fractionated yeast membranes on urografin gradients, and assayed the ability of fractions to transfer mannose from GDP-mannose to O-linkage to

polypeptides. They found the highest specific activity for addition of the first mannose residue in the endoplasmic reticulum, whilst addition of 1-3 more residues occurred with higher specific activity in Golgi and plasmalemma enriched fractions, suggesting sequential addition of mannose residues. O-glycosylations in fungi are, however, unique in using dolichol phosphate-mannose as initial mannose residue donor (Sharma et al., 1974; Bretthauer and Wu, 1975). Johnson and Spear (1983) found that O-glycosylation of herpes simplex virus glycoproteins in mammalian cells occurred entirely in the Golgi apparatus. There is evidence that in mammalian cells different sugars are added to the growing oligosaccharide in different Golgi cisternae (Elhammer and Kornfeld, 1984; Roth, 1984).

The recognition sequences for sites of O-glycosylation are unknown. Hill et al. (1977) found no sequence homologies adjacent to 28 sites of O-glycosylation of ovine submaxillary mucin. Lehle and Bause (1984) found no marker sequence in studies of O-glycosylation of synthetic peptides by yeast membranes, although a proline residue in the vicinity of the glycosylation site increased efficiency of transfer, suggesting that accessibility might be an important factor. Conformational modelling based on the complete amino acid sequence of human casein demonstrated that 9 of 10 O-glycosylated threonine residues are in regions with a high capacity to form beta-turns, whilst four non-glycosylated threonine residues are in regions with greater capacity to form other structures (Aubert et al., 1976; Fiat et al., 1980).

The physiological function of protein glycosylation remains an enigma. Of the variety of functions proposed, stabilisation and protection against proteolysis appear significant in secreted proteins. Chu et al. (1978) demonstrated that removal of 90% of the carbohydrate from yeast invertase by endoglycosidase H did not alter the catalytic or physical properties of the enzyme, but rendered it much less stable to multiple freeze-thaw cycles, heating, low pH and trypsin treatment. Upon denaturation by guanidine chloride followed by removal of this reagent, the glycosylated form regained 76% of its

initial activity. The activity regained by deglycosylated invertase was only 34%. Circular dichroism and tryptophan fluorescence spectroscopy indicated that refolding of the deglycosylated protein was not to the native conformation. Other postulated functions of glycosylation are discussed in the next section.

The nature of the carbohydrate moieties of the glycoproteins detected in the current experiments, and the protein-carbohydrate linkages are unknown. Whilst hydroxyproline-rich glycoproteins and arabinogalactan proteins, which exhibit extensive O-glycosylation are extracellular molecules, N-glycosylation is found in plants in several seed storage proteins, including soya bean and lima bean agglutinins, as well as the enzymes alpha-amylase, bromelain and horseradish peroxidase. Mixed N- and O-glycosylation of a single protein has been observed in animals, but not plants (Sharon and Lis, 1979). That no amino sugars were detected in the amino acid analysis of the 16.4B4 antigen (Chapter 6) suggests that oligosaccharides are O-linked, since N-linked glycosyl moieties are attached to the polypeptide via proximal N-acetylglucosamine residues. Analogy with other plant proteins may also indicate the nature of the 16.4B4 antigen, and hence its expected pattern of glycosylation. In the next section, similarities are discussed between the 16.4B4 antigen, extracellular arabinogalactan proteins of plants, and surface localised glycosaminoglycans of animal cells.

Does 16.4B4 recognise an arabinogalactan protein?

Considerable information has been accumulated as to the properties of the 16.4B4 antigen. It appears that the antigen has a central protein core of 50 kd or less molecular weight, as judged by the deglycosylation studies recorded in Chapter 6. This is extensively and heterogeneously glycosylated, increasing the apparent molecular weight to 130-230 kd. Such extensive glycosylation is not characteristic of glycoproteins, which are usually glycosylated at a few discrete residues, and show more limited molecular weight heterogeneity. More extensively glycosylated proteins are usually classed as



proteoglycans, which differ from glycoproteins in containing longer polysaccharide substituents, rather than the shorter oligosaccharide moieties found in the latter. Many plants have been found to contain representatives of a class of proteoglycan known as arabinogalactan proteins (AGPs).

The structural characteristics of AGPs have been extensively reviewed by Clarke *et al.* (1979) and by Fincher *et al.* (1983). Most are highly soluble, and can be isolated from aqueous extracts of plant tissues or from tissue culture filtrates. They include the gums, which are commercially important plant exudates. Of the many AGPs isolated from plant cells, most contain only 2-10% protein, although in some the protein content is higher, eg: 59% in *Acacia hebecclada* gum (Anderson and Farquhar, 1979). The balance of the molecule is polysaccharide, which is typically rich in D-galactopyranose and L-arabinofuranose, with ratios between the two of 1:9 to 17:3 (Fincher *et al.*, 1983), although other monosaccharide residues may make up a significant proportion of some AGPs. Polysaccharide components are branched, having mostly beta-1,3- but also beta-1,6-linkages (Gleeson and Clarke, 1979), whilst viscosity measurements in solution suggest a spherical shape for several AGPs (Swenson *et al.*, 1968; Adams and Ettling, 1974). Glycosylation of the protein core is principally via O-linkage to serine or hydroxyproline residues, although threonine linked carbohydrate has also been detected (Van Holst and Klis, 1981). N-linked carbohydrate has not been detected.

A diagnostic feature of AGPs is their ability to react with Yariv antigens with beta-D-glucosyl substituents, resulting in the precipitation of an AGP complex (Yariv *et al.*, 1967; Jermyn and Yeow, 1975). The nature of this interaction remains obscure. The finding of Larkin (1977) that beta-glucosyl Yariv antigens could aggregate protoplasts of a variety of plant species and organs implies that AGPs are associated with the plasmalemma. Precipitation with Yariv antigens was not attempted in the present study, and the carbohydrate composition of the 16.4B4 antigen was not determined, but less direct evidence supports the contention that this antigen might be an AGP.

Amino acid compositional analyses have been reported for a number of AGPs. Characteristically, these define the protein core as rich in hydroxyproline, serine, alanine and glycine-properties which are shared by the 16.4B4 antigen. Table 9.1 summarises published amino acid analyses for a number of AGPs and animal cell proteoglycans (the latter will be discussed later). There is considerable similarity in composition between the different AGPs and the 16.4B4 antigen. In particular, the compositions reported by Hori and Sato (1977), by Akiyama and Kato (1981) and by Akiyama *et al.* (1982) for AGPs from *N. tabacum* are similar to that of the 16.4B4 antigen. In the former studies, AGPs were isolated from *N. tabacum* suspension culture filtrates. The differences in the reported amino acid compositions may be the result of use of different strains (Xanthi and Bright Yellow respectively). Akiyama *et al.* (1982) isolated an AGP by treatment of *N. tabacum* leaves with 1% sodium deoxycholate solution, which extracted essentially all Yariv antigen precipitable material. The purified AGP contained arabinose, rhamnose and galactose in the molar ratio 1:0.22:1.25, as well as glucuronic acid, xylose, glucose and mannose residues. The molecular weight as estimated by gel filtration was 220 kd. The AGP did not require the presence of detergent to maintain it in solution once isolated, and so was probably not membrane bound but intracellular. Such intracellular AGPs have been reported in a number of species (Anderson *et al.*, 1977).

In contrast, although it has a similar amino acid composition, little of the 16.4B4 antigen is extracted by TBS, the bulk requiring detergent extraction and the continued presence of detergent to maintain it in solution (Chapter 6). The AGPs detected by Larkin (1977) on the exterior of the plasmalemma must be intimately associated with that membrane, and so are more likely to be analogous to the 16.4B4 antigen.

Table 9.1. Published amino acid compositions of several plant and animal proteoglycans.

Amino acid	16.4B4 antigen	N. tabacum cum leaf	N. tabacum suspension culture exudate	Wheat endo-sperm	P. vulgaris secreted	Cannabis sativa leaf	Chick embryo cartilage	Rat yolk sac tumour PM	Rat liver PM	Ascites hepatoma PM
Ala	13.9	15.6	21.3	13.0	18.0	16.2	10.4	6.8	5.7	6.9
Arg	2.1	0.8	--	1.2	2.3	0.6	--	1.5	2.1	3.3
Asp	8.4	7.0	3.3	9.9	6.6	3.9	13.3	8.8	12.5	13.0
Hyp	20.0	20.0	23.8	16.5	11.5	26.0	--	--	--	--
Cys	0.6	--	--	--	--	0.6	--	--	--	--
Glu	11.5	5.6	5.3	9.2	15.9	4.5	15.9	11.6	16.1	14.4
Gly	14.7	10.5	5.8	7.1	4.2	6.4	8.6	18.5	10.0	9.2
His	0.1	2.1	trace	1.1	1.0	0.6	--	6.1	1.2	--
Ile	3.0	2.0	0.9	1.8	2.6	1.3	4.0	1.8	3.4	2.1
Leu	5.1	3.2	1.9	3.5	3.1	3.2	7.6	2.0	6.8	7.5
Lys	1.4	1.4	2.1	4.0	3.6	2.5	--	2.2	4.1	2.6
Met	0.9	0.8	0.5	1.2	1.9	0.6	2.4	1.0	1.5	--
Phe	2.2	1.7	--	1.9	1.2	0.6	2.7	4.3	1.6	4.8
Pro	4.7	3.6	5.3	4.9	3.9	2.6	7.0	6.1	7.7	11.7
Ser	16.2	13.4	14.8	10.7	8.6	18.2	9.7	18.3	14.2	9.2
Thr	7.8	8.7	9.3	6.0	6.0	7.1	8.7	3.4	7.8	11.1
Tyr	1.8	0.6	trace	4.0	2.9	0.6	3.1	--	1.9	0.5
Val	5.5	3.6	5.5	4.0	5.8	0.3	6.7	7.2	3.3	3.7
				ornithine	0.6	hydroxylysine	1.0			

Ref:

1	2	3	4	5	6	7	8	9	10
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References:

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2. Hori and Sato (1977).
3. Akiyama and Kato (1981).
4. Strahm et al. (1981).
5. Van Holst et al. (1981).
6. Hillestad et al. (1977).
7. Noro et al. (1983).
8. Oldberg et al. (1981).
9. Oldberg et al. (1979).
10. Mutoh et al. (1978).

Fincher et al. (1983) pointed out the similarity in properties between AGPs and the animal proteoglycans, known as glycosaminoglycans (GAGs). The GAGs have been studied in some detail with respect to their localisation, interactions and physicochemical and structural properties (reviewed by Hook et al., 1984). GAGs have been classified according to their carbohydrate structures, which are chains of alternating disaccharides of different composition for each class. For example, keratin sulphates contain alternating beta-D-galactose and beta-D-glucosamine residues, whilst heparan sulphates and heparin contain alpha-D-glucosamine residues alternating with beta-D-glucuronic acid or alpha-L-iduronic acid residues. In addition, there may be substantial O-sulphation of sugar residues after synthesis of the GAGs. Although the sugar composition of AGPs differs from that of GAGs, and no post-synthesis modification of the former has been demonstrated, the two classes show similarity in the composition of their protein cores. Table 9.1 shows published amino acid analyses for a number of GAGs. Characteristically, these show high glutamate, serine and glycine contents, as do AGPs. GAGs of a single class may contain core proteins of more than one type. For example, the chondroitin sulphate proteoglycan from cartilage has a heterogeneous core protein of 200-250 kd. In vitro translation of the mRNA yields a primary transcript of 340 kd (Upholt et al., 1979; Treadwell et al., 1980), which is presumed to contain a signal sequence which is cleaved during maturation (Kimura et al., 1981).

Heparan sulphate proteoglycans of rat hepatocytes are of two classes: one has a core protein of molecular weight 27 kd, the other of 35 kd (Kjellen and Hook, 1983). The properties of this proteoglycan have interesting similarities to those of the 16.4B4 antigen. Proteoglycans were initially isolated from the extracellular matrix of connective tissues (AGPs are found in the middle lamella-a similar structure), but are also found at cell surfaces. Kjellen et al. (1980) showed that two thirds of the hepatocyte cell-bound heparan sulphate could be displaced by exogenous heparan sulphate or heparin, indicating a ligand-receptor type association with the plasmalemma. In contrast, the remaining third required trypsin treatment for release.

Kjellen et al. (1981) showed that this population could also be released by detergent treatment. Heparan sulphates of the two pools were identical in molecular weight, but the detergent extractable proteoglycan had a lower buoyant density than that which was heparin displaced. Detergent soluble heparan sulphate could be reassembled into liposomes. The purified proteoglycan had a molecular weight of ca. 75 kd, and was composed of the core protein with four 14 kd polysaccharide chains attached (Oldberg et al., 1979). These properties are rather similar to those of the 16.4B4 antigen, of which a fraction is buffer soluble, whilst a greater amount is solubilised by detergent treatment. It was suggested that the peripheral hepatocyte proteoglycan was derived from the intercalated form by a proteolytic cleavage (Kjellen and Hook, 1983); a similar processing event might account for the two solubility classes of the 16.4B4 antigen.

A number of immunofluorescence microscopy studies have been performed using antibodies to surface associated GAGs of animal cells (eg: Hedman et al., 1983; Oldberg et al., 1981). These show rather similar immunofluorescence images to those found for antibody 16.4B4 (Chapter 4), with intense continuous staining of the plasmalemma. Polyacrylamide gel electrophoresis of radiolabelled purified proteoglycans, or electrophoresis followed by Western blotting (Noro et al., 1983; Mutoh et al., 1978) also show rather similar staining patterns to those seen on Western blots of the 16.4B4 antigen (Chapter 5). Proteoglycans were detected as diffuse, high molecular weight bands, showing considerable molecular weight heterogeneity. This molecular weight heterogeneity is probably due to microheterogeneity of glycosylation and sugar residue sulphation. The processes controlling the extent of glycosylation and length of attached polysaccharide chains are unknown.

Unfortunately, the similarity between the 16.4B4 antigen and animal and plant proteoglycans does not suggest a function for the former, since neither for AGPs nor GAGs is a function established. Since AGPs display a variety of polysaccharide residues, they could

interact with lectins, or with other proteins or polysaccharides, such as pectins in the middle lamella (Fincher et al., 1983). GAGs are known to interact with other macromolecules in the extracellular matrix. For example, in cartilage many chondroitin sulphate proteoglycans interact via their core protein with a single hyaluronic acid molecule to form large aggregates (Hardingham and Muir, 1973; Hascall and Heinegard, 1974; Christner et al., 1979). There are similar interactions with collagen (Hascall and Hascall, 1981), and with fibronectin (Laurie et al., 1983; Leivo, 1983). The enzyme lipoprotein lipase is displaced from the surface of endothelial cells by addition of exogenous heparan sulphate, suggesting that it is bound to cell associated heparan sulphate in vivo (Cheng et al., 1981). AGPs have been localised to vesicles in the intercellular spaces between parenchyma cells (Clarke et al., 1978), in the stylar canal of Lilium (Loewus and Labarca, 1973), and at the cytoplasm-wall interface in the aleurone layer of cereal seeds (Anderson et al., 1977). It is thus possible that the 16.4B4 antigen interacts with extracellular macromolecules. The most immediately accessible of these are in the cell wall, but the soluble form of the antigen might be in transit to the extracellular spaces, where AGPs are known to accumulate (Akiyama and Kato, 1981). This might imply a structural role for the antigen.

Alternatively, the 16.4B4 antigen could interact with low molecular weight ligands. Since AGPs interact with Yariv antigens and flavonol glycosides (Jermyn, 1978), they themselves have lectin-like properties, and could interact with oligosaccharides. Plasmalemma associated AGPs would have the potential to convey this information to the cytoplasmic face of the plasmalemma. There is no evidence that AGPs or GAGs act as receptors of external stimuli, whilst recently it has been shown that self-incompatibility in Cruciferae and Solanaceae is associated with expression of a glycoprotein rather than an AGP (Nasrallah et al., 1985; Anderson et al., 1986). Nevertheless, the ability to recognise specific oligosaccharides has been postulated to be important in morphogenesis (Thanh Van et al., 1985) and disease resistance (Darvill and Albersheim, 1984) in higher plants. Whether AGP-oligosaccharide interactions have the specificity necessary to

these interactions is unknown.

The terminal disaccharides of plant gums show taxonomic differences (Anderson and Dea, 1969). It has been postulated that AGPs might be involved in the expression of species, tissue or cell identity in plants, in a similar manner to the oligosaccharide moieties of blood group and transplantation antigens in man (Fincher et al., 1983).

Cell density dependent changes in mammalian cell GAG type and rate of synthesis have been recorded. It was found that the chondroitin sulphate content of normal mouse 3T3 cells increased with culture density, whilst in SV40 transformed cells it was density independent (Underhill and Keller, 1976; Cohn et al., 1976). Vannucchi and Chiarugi (1977) found that growing and SV40 transformed 3T3 cells show similarly low levels of heparan sulphate and high expression of hyaluronic acid. In resting untransformed cells, the ratio was reversed. Some difference was seen in the level of expression of the 16.4B4 antigen through the growth cycle of *N. glutinosa* cells in culture (Chapter 8). Although this was not pronounced, the level decreased during active growth of the cells, and increased again as the static phase was reached at the end of the growth cycle (Fig. 6.8).

It has also been suggested that AGPs may be important in frost tolerance (Williams, 1973), wound healing and resistance to desiccation (Clarke et al., 1979), although their water holding capacity is probably insufficient for this purpose (Fincher et al., 1983).

Apart from their involvement in extracellular matrices, no function has been assigned to AGPs or GAGs. The possible function of plasmalemma associated proteoglycans is thus obscure. If the 16.4B4 antigen is indeed a plasmalemma associated AGP, this represents the first isolation of such a molecule, but provides no clues as to its function. As has been noted (Lindhahl et al., 1986; McNeil et al.,

1984), structure-function relationships of complex carbohydrate containing molecules are in general poorly understood.

The nature of the plant plasmalemma.

The work recorded in this thesis allows a number of conclusions to be drawn as to the nature of the plasmalemma, and of its constituents in *N. glutinosa*. Although these studies have been focussed on suspension cultured cells, there is considerable evidence that the plasmalemma has similar properties in intact plants (results of M.S. Fitter, M.G. Hahn, D.R. Lerner and P.M. Norman, not shown). Isolation of the plasmalemma from plant cells is rendered difficult by its intimate association with the cell wall. Thus its isolation from intact cells requires application of high shear forces to rupture the wall (Galun, 1981), in addition to which a substantial portion of the membrane probably remains associated with cell wall debris after homogenisation and is lost from the membrane preparation (Hendriks, 1978b, Lembi *et al.*, 1971; Nagahashi and Beevers, 1978). In contrast, protoplasts have the advantage of having the wall removed enzymically. Although this has advantages over homogenisation of intact cells, it should not be concluded that the procedure is "gentle". Protoplasting takes from 45 min (Nagata *et al.*, 1981) to 48 h (Haberlach, 1985), and is accompanied by shock to the cell, both due to the increased osmotic potential of the medium, and to the process of digestion itself (Fleck *et al.*, 1982). These factors alter cellular metabolism, and hence may directly or indirectly change the properties of the plasmalemma. In addition, the cellulolytic enzymes are impure, and may contain activities which can digest plasmalemma components, such as proteins and oligosaccharide chains. The fragility of protoplasts will also reduce the yield of cells and hence plasmalemma compared to intact tissues.

Despite these potential problems, it has proved possible to use a heterogeneous membrane preparation to generate antibodies which recognise plasmalemma constituents. The immunofluorescence microscopy screening of monoclonal antibody specificity indicates that the



plasmalemma possesses antigenic determinants which are unaffected by protoplasting, and that antibodies which react with the external face of the plasmalemma were generated with rather high frequency. Subsequent hybridoma production indicates that this was probably serendipitous (M.S. Fitter and D.R. Lerner, unpublished results), although as discussed above, the extensive glycosylation and possible immunodominance properties of plasmalemma associated antigens might be significant.

Slot blot assays on membrane fractions allow estimation of the fractions containing peak amounts of plasmalemma expressed antigen, although differences in sidedness of plasmalemma vesicles and the potential intracellular expression of antigenic determinants associated with the external face of the plasmalemma may affect the results (Chapter 7). These potential problems are circumvented by the topological labelling procedure, in which all of the antigenic determinant associated with the external face of the plasmalemma is available for antibody binding, whilst all intracellular antigen in intact protoplasts is inaccessible to the antibody. The bound antibody will be detected after homogenisation regardless of sidedness of the vesicles with which it is associated. This procedure results in assignment of the plasmalemma to a single peak with a maximum density of 1.14 kg/l.

These studies pave the way to a rigorous molecular characterisation of the plasmalemma. First, using monoclonal antibody markers, it will be possible to derive optimum separation strategies for the plasmalemma, without recourse to the ambiguous enzymatic, ligand binding and vesicle staining assays usually employed (see Chapter 1). Purification methods based on combined two phase and sucrose density gradient separation have been described (Yoshida et al., 1983, 1986), and are currently under development for the purification of the plasmalemma of N. glutinosa using monoclonal markers (M.G. Hahn, unpublished results). There is a published report of isolation of plasmalemma from rat hepatocytes using immunoaffinity chromatography (Devaney and Howell, 1985), a method which might prove useful in this

system. Such techniques should allow the constituents of the plasmalemma to be isolated and characterised, both from Nicotiana and from commercially important but biochemically poorly characterised crop plants, such as rice and wheat, since several plasmalemma specific monoclonal antibodies cross-react with protoplasts from these species (M.S. Fitter, unpublished results).

Second, new hybridoma libraries can be generated to highly purified plasmalemma, which should increase the range of surface reactive monoclonal antibody specificities available. Deglycosylation or drug induced antigen tolerance (Matthew and Patterson, 1983) could increase the heterogeneity of antibody reactivity, by elimination of immunodominant specificities. In addition, plasmalemma composition can be compared between plant strains and tissues with different properties (eg: regenerant versus non-regenerant cell lines, cultivars resistant and susceptible to pathogens, leaf versus root tissues). Many of these properties have been postulated to be associated with plasmalemma components (Quail, 1979; Hall, 1983). Any differences between plasmalemma properties would be more likely to be detected using a purified membrane. Methods exist for efficient production of hybridomas using very small quantities of immunogen (Luben et al., 1982; Lo et al., 1984). Such antibodies may be used to purify and characterise the antigen, and for isolation of protoplasts carrying a particular surface antigen using fluorescence activated cell sorting.

Third, monoclonal antibodies provide a method for isolation of the gene encoding a particular polypeptide. The immunoaffinity purified polypeptide may be microsequenced, and a complementary oligodeoxy-ribonucleotide synthesised as a probe of a cDNA library, as described by Anderson et al. (1986). Alternatively, polyclonal antisera may be generated and used to screen a cDNA library in an expression vector, as described by Young and Davies (1983). Such methods will allow the examination of amino acid sequences responsible for targetting a particular polypeptide to the plasmalemma, manipulation of the fate of the polypeptide and examination of the effect of the altered fate on plant physiology, and perhaps ultimately alteration of the

susceptibility of a plant to disease, or of the amenability of a species to genetic manipulation and regeneration to fertile plants.

APPENDIX.

ARTIFACTUAL ANTIBODY BINDING TO STEM SECTIONS.

## INTRODUCTION.

Jacobs and Gilbert (1983) reported the production of monoclonal antibodies which inhibit the binding of naphthylphthalamic acid to pea stem cell homogenates. They presented immunofluorescence microscopic evidence that the antigen recognised by one of these antibodies was located at the basal end of stem cells, in support of the chemiosmotic hypothesis of polar auxin transport (Rubery and Shelldrake, 1974). It seemed unlikely that the conditions under which this experiment was performed were suitable for the preservation of intracellular structure of cut cells, or for the penetration of antibody to the plasmalemma of uncut cells. For this reason, the experimental procedure was repeated, using plasmalemma specific and control antibodies from the present library. The evidence suggests that the observed polar binding of monoclonal antibodies under these conditions is artifactual.

## MATERIALS AND METHODS.

Antibody labelling and immunofluorescence microscopy of stem sections of etiolated pea seedlings was performed as described by Jacobs and Gilbert (1983). Seedlings of Pisum sativum cv. Alaska were grown from seeds in the dark for 7 days. Median longitudinal sections were hand cut with a razor blade from the third internode, to be ca. 1 mm long and tapering towards either the top or bottom of the section. Following rinsing in distilled water, they were incubated for 45 min at room temperature with neat supernatants from hybridomas 16.1B3, 16.4B4, and 17.3B4, as well as VC40.2B2 and unused DMEM as controls. Sections were washed three times in PBS containing 0.5% (w/v) BSA, then incubated for 45 min with fluorescein conjugated goat anti-mouse antibody (HyClone, Logan, Utah) diluted 1:100 in the same solution. The sections were washed three times as above, mounted in 50% (v/v) glycerol in PBS, and viewed under epifluorescence microscopy as described (Chapter 4).

## RESULTS.

Results of immunofluorescence microscopy on stem sections are shown in Figure A.1. Monoclonal antibodies 16.1B3 and 17.3B4 showed bright fluorescence at the walls of cut cells, whilst 16.4B4 showed a similar level of fluorescence to sections treated with DMEM or VC40.2B2. Whereas the positive binding was evenly distributed throughout the section, background binding showed clear polarity in the section. The direction of polarity depended on the direction of cutting of the section. For sections tapering towards the base of the stem, antibody was localised towards the apex of the section. For sections tapering in the other direction, the polarity of fluorescence was reversed. No effect on the labelling was seen if azide was included in the buffers, indicating that localisation was not dependent on metabolic processes.



Figure A.1. Binding of monoclonal antibodies to pea stem sections.

Hybridoma supernatants used were (A) DMEM, (B) VC40.2B2, (C) 16.1B3, (D) 16.4B4 and (E) 17.3B4.



## DISCUSSION.

The results of this experiment suggest that specific antibody binding to the plasmalemma is unlikely to be seen under the conditions described by Jacobs and Gilbert (1983). The use of phosphate buffered saline rather than an osmotically active solution is likely to result in lysis of any cells, of which the plasmalemma was exposed in cutting the section. In contrast, antibody would not be expected to reach the plasmalemma of intact cells. The observed polar fluorescence in the sections treated with DMEM, VC40.2B2 or 16.4B4 is probably due to trapping of antibody by debris in the upper side of the section, where it will be less susceptible to washing out by buffers. A positive signal, such as observed for 16.1B3 or 17.3B4 is considerably brighter, and in neither case shows polarity. That Jacobs and Gilbert found no fluorescence in pea stem sections using inactive or irrelevant antibodies remains unexplained, but could be due to failure to section cells at an angle, which would result in the sections having no polarity.

As discussed in the introduction, these results suggest that the evidence in support of the chemiosmotic theory of polar auxin transport, deduced by Jacobs and Gilbert (1983) from similar experiments, is probably not valid. An accurate assessment of the sites of binding of the monoclonal antibody in intact cells would probably require the use of immuno-electron microscopy. It seems unlikely that hand sectioning is a valid method of determination of sites of antibody binding in stem sections.

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